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PATENT TRADEMARK OFFICE

Docket No: 1225/0C674

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David BERD

Serial No.: 08/203,004 Art Unit: 1642

Confirmation No.:

Filed: February 28, 1994 Examiner: Susan UNGAR

For: COMPOSITION AND METHOD OF USING TUMOR CELLS

BRIEF ON APPEAL

December 28, 2001

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

This Brief on Appeal (submitted in triplicate) follows the Notice of Appeal filed May 29, 2001 and the response to the Final Office Action mailed on November 29, 2000. Appellant submits concurrently herewith (1) a Petition for Extension of Time for a period of five months (from July 29, 2001 up to and including December

31, 2001, as December 29 falls on a Saturday) accompanied by the required fee; and (2) the required fee for this Brief. It is believed that no additional fees are required for these submissions. However, should it be determined that additional fees are required or that any refund is due in connection with this application, the Commissioner is hereby authorized to charge the required fee(s) and/or credit the refund(s) due to Deposit Account No. 04-0100.

1. The Real Party in Interest

Thomas Jefferson University (TJU), Philadelphia, Pennsylvania, is the assignee of this application. Avax Technologies, Inc., of Overland Park, Kansas (Avax), has an exclusive license from TJU. Accordingly, Avax is a real party in interest.

2. Related Appeals and Interferences

There are no related appeals or intereferences.

3. Status of Claims

Claims 43, 44, 47, 49-62, 64-72, and 74-77 are pending and the final rejection of all of these claims is the subject of this Brief. A copy of the pending claims is attached as an Appendix immediately following this Brief.

4. Status of Amendments

Appellant filed an amendment May 29, 2001 in response to the Final Office Action dated November 29, 2000. The Examiner entered this amendment pursuant to the Advisory Action dated July 5, 2001 (copy attached as Exhibit 1).

5. Summary of the Invention

The present invention concerns a composition comprising human tumor cells (other than melanoma cells) conjugated with a hapten.[1] Such haptenized tumor cells have been surprisingly and unexpectedly discovered to form an effective immunogenic component in a vaccine composition for immunotherapy of cancer of the type from which the cells were derived. The haptenized tumor cells are obtained from the patient receiving treatment (i.e., they are "autologous"), and are rendered incapable of growing in the body of a human upon injection therein.

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[1] A hapten is a small molecule that, when conjugated to a carrier, can elicit a specific immune response. Preferred haptens include the highly reactive dinitrophenyl and trinitrophenyl groups. (Specification, page 15, lines 4-9).

In another aspect, the invention provides a method for treating a malignant tumor (other than melanoma) in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant. The composition elicits at least one of the following upon administration to the patient with the adjuvant: an inflammatory immune response against the tumor of the patient; a delayed-type hypersensitivity response against the tumor of the patient; and activated T lymphocytes that infiltrate the tumor of the patient.

In a further aspect, the invention provides a method for treating a malignant tumor in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant, at least six times. In still a further embodiment, the patient receives a dose of cyclophosphamide prior to the first administration of the composition.

The composition of the invention represents an advance over prior experiments involving haptenization of tumor and other cells for testing in animal models. Prior art experiments suggested that haptenization results in hapten-specific immunity. Such immunity would not proved useful for generating an effective immune response against metastasized tumor cells or tumor cells remaining after tumor resection because the residual tumor cells in a patient do not bear hapten.

The invention addresses a need in the art for an effective immunotherapy for tumors, especially non-melanoma tumors. Administering haptenized tumor cells unexpectedly increases the effectiveness of the resulting tumor-specific immune response, especially with six or more immunizations, resulting in a more effective immunotherapy. Most importantly, the inventor has discovered that the protective immunity is not hapten-specific, which the prior art suggested would be the case.

6. Issues

The only remaining issues in this application concern obviousness of the claims over various combinations of references as set forth below.[2]

a. The rejection over Murphy, Berd 1989, Geczy, and the Antibody Patents: Claims 47, 65-72 and74-77 stand rejected as allegedly being obvious (see

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[2] The Examiner withdrew the rejection of claims 43, 49-51, and 54-55 for allegedly not being enabled by the disclosure in the Advisory Action (Exhibit 1) in view of the amendment of claim 43 to recite that the composition of the invention elicits, when administered together with an adjuvant, an immune response. In a previous Office Action, the Examiner stated that the specification enables a method for treating a malignant tumor in a human patient comprising administering the composition of claim 43 (*i.e.*, haptenized autologous non-melanoma tumor cells) and BCG (Office Action dated April 28, 1999 [Exhibit __; Paper No. 36], paragraph No. 6). The specification supports this recitation, e.g., Examples 2 and 3 report eliciting a striking inflammatory response when the composition of the invention was administered together with the adjuvant BCG. Claims 49-51 and 54-55 depend from claim 43.

paragraph No. 5 of the Final Office Action [Exhibit 2], referencing Paper No. 41 [Exhibit 3], Section 5, pages 2-3 and Paper No. 36 [Exhibit 4], Section 10, pages 8-12) over Murphy et al. (Lab Invest 1990;62:70A; hereinafter "Murphy" [Exhibit 5]), in view of U.S. Patent No. 5,702,704 (hereinafter "'704 patent" [Exhibit 6]), U.S. Patent No. 5,626,843 (hereinafter "'843 patent" [Exhibit 7]), U.S. Patent No. 5,008,183 (hereinafter "'183 patent" [Exhibit 8]), or U.S. Patent No. 4,232,001 (hereinafter "'001 patent" [Exhibit 9]) (hereinafter collectively "the Antibody Patents"); Berd *et al.*, (Proc AACR 1989:30:382; hereinafter "Berd 1989" [Exhibit 10]), and Geczy et al. (J Immunol. 1970;19:189-203, hereinafter "Geczy" [Exhibit 11]).

b. The rejection over Berd 1989, the Antibody Patents, and Geczy: Claims 47, 65-72, and 74-77 stand rejected as allegedly being obvious over Berd 1989 in view of the Antibody Patents and Geczy (see paragraph 6 of the Final Office Action [Exhibit 2], referencing Paper No. 41 [Exhibit 3], Section 6, page 4 and Paper No. 36 [Exhibit 4], Section 11, pages 12-15).

c. The rejection over Berd 1989, the Antibody Patents, and Geczy in view of Wiseman: Claims 43, 44, 47, 49-62, 64-72, and 74-77 stand rejected as allegedly being unpatentable over Berd '89 in view of the Antibody Patents, and Geczy, in further view of Wiseman et al. (West J Med 1989;151:283-288, hereinafter "Wiseman" [Exhibit 12]) (see paragraph 7 of the Final Office Action [Exhibit 2],

referencing Paper No. 41 [Exhibit 3], Section 7, pages 4-5 and Paper No. 36 [Exhibit 4], Section 12, pages 15-18).

d. The rejection over Berd 1989, the Antibody Patents, Geczy, and Berd 1983: Claims 43, 44, 47, 49-62, 64-72, and 74-77 stand rejected as allegedly being unpatentable over Berd '89 in view of the Antibody Patents, and Geczy, in further view of Berd et al. (PASCO 1983;2:56, hereinafter "Berd 1983 [Exhibit 13]) (Final Office Action [Exhibit 2], paragraph No. 8, referencing Paper No. 41 [Exhibit 3], Section 8, page 6 and Paper No. 36 [Exhibit 4], Section 13, pages 18-21).

e. The rejection over Berd, the Antibody Patents, Geczy, and Sanda and Moody: Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected as allegedly being unpatentable over Berd '89 in view of the Antibody Patents, Geczy, in further view of Sanda et al. (J Cellular Biochem 1993;suppl.17D:120, hereinafter "Sanda" [Exhibit 14]) and Moody et al. (J Urol 1991;145:293A, hereinafter "Moody" [Exhibit 15]) (Final Office Action [Exhibit 2], paragraph No. 9, referencing Paper No. 41 [Exhibit 3], Section 9, page 7 and Paper No. 36 [Exhibit 4], Section 14, pages 21-25).

7. Grouping of Claims

The claims do not stand or fall together. Claims 43 and 49-55 are directed to compositions that have distinct features and patentability considerations. Within this group, claim 49 is directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 51 is directed to a specifically recited hapten.

Claims 44, 56-62, 64, and 76 are directed to a method for treating a malignant tumor in a human, which has distinct patentability considerations relative to the composition claims. Within this group, claims 56 and 57 are directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 59 is directed to a specifically recited hapten.

Claims 47, 65-72, 74, 75, and 77 are directed to a method for treating a malignant tumor in a human, which has distinct patentability considerations relative to the composition claims and to the other method of treatment claims because these claims (i) do not exclude treatment of melanoma tumors and (ii) require at least six administrations of the immunotherapy vaccine. The Examiner has rejected these claims for different reasons than the other claims, which further establishes that these claims stand or fall separately from the other claims. Within this group, claims 65 and 66 are directed to a Markush group of tumors that has distinct patentability considerations relative to the genus of tumors. In addition, claim 68 is directed to a specifically recited hapten. Finally, claim 70 recites a specific regiment for administration of cyclophosphamide (CY).

8. Argument

The relevant test for obviousness requires three basic factual inquiries: the scope and content of the prior art are to be determined; the differences between the prior art and the claims at issue are to be ascertained; and the level of ordinary skill in the art resolved. *Graham v. Deere*, 383 U.S. 1, 17 (1966); *Ruiz v. A.B. Chance Co.*, 57 USPQ2d 1161, 1165 (Fed. Cir. 2000). The relevant inquiry involves three steps. First, there must be some suggestion or motivation to modify what is taught in a reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference or combination of references must teach all of the claim limitations. Both the motivation and the reasonable expectation of success must be found in the prior art, not in appellant's disclosure. *See*, MPEP § 2143, *citing In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

As explained in detail below, the Final Office Action fails to establish a *prima facie* case for obviousness under these requirements. In particular, since Berd 1989 fails to provide any expectation of success, *i.e.*, clinical benefit, using the haptenized tumor cell approach in melanoma patients, this reference is completely irrelevant in providing any expectation of success for such an approach in other types of cancer. Since no other reference cited by the Examiner makes up for this

fundamental flaw, nor the combination of them (see below), obviousness does not obtain.

Given the prominence of Berd 1989 in each rejection, the Appellant would like to address the teachings of this reference, particularly through the lense of one of ordinary skill in the art, Dr. Donald Braun, who attended an interview with the Examiner and her supervisor on January 5, 2000.[3] To factually determine what a reference teaches one of ordinary skill in the art in implementing the Graham standard, the courts have relied upon affidavit evidence either by experts or those of ordinary skill in the art. *See In re Carroll* 202 USPQ 571 (CCPA 1979); *In re Piasecki*, 223 USPQ 785, 789 (Fed. Cir. 1984); *In re Oelrich*, 198 USPQ 210 (CCPA 1978). Furthermore, affidavits of those skilled in the art have been held to constitute factual evidence of the level of skill in the art. *E.g., In re Piasecki*, 223 USPQ at 789; *In re Oelrich*, 198 USPQ 210, 214-15. Such affidavits constitute competent evidence that cannot be ignored. *See e.g., Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.*, 227 USPQ 657, 674-75 (Fed. Cir. 1985).

In all of the rejections, the Examiner relies on the Berd 1989 abstract as an allegedly successful example of treatment of melanoma by administration of DNP-conjugated autologous melanoma cells in connection with BCG and a preceding dose

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[3] Dr. Donald Braun has a long career in the field of immunological oncology, as evidence by his *curriculum vitae*, attached as Exhibit A to the Braun Declaration [Exhibit 16]. There can be no doubt as to his qualifications as one of at least ordinary skill in the art.

of cyclophosphamide. The Examiner also contends that it would have been expected that the autologous irradiated melanoma, lung, colon, kidney, and colon cancer cells of Wiseman ([Exhibit 11] discussed below) would be successfully substituted for the melanoma cells of Berd 1989 to treat other cancer types (Final Office Action [Exhibit 2], bridging paragraph between pp. 5 and 6). However, both of these conclusions depend on according more weight to the Abstract than one of ordinary skill at the time of the invention would have given it. As set forth by the Braun Declaration accompanying the response to the Final Office Action[4], Berd 1989 does not describe a successful immunotherapy for melanoma (Braun Declaration [Exhibit 16], paragraph 7). On the contrary, it represents a preliminary result that raises more questions and ambiguities than it answers. Early animal work on tumor immunotherapy could not establish whether similar approaches could work in humans (Braun Declaration [Exhibit 16], paragraph 8). The Abstract fails to provide a definitive protocol that would permit one to repeat the work, determine whether this approach elicited an immune response

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[4] The Braun Declaration memorializes comments made by Dr. Braun during THE personal interview with the Examiner and her Supervisor on January 5, 2001, at which this application and a number of related applications by the same inventor were discussed. The points made therein reinforce scientific and factual argument distinguishing the prior art of record already made by applications. The Examiner agreed at the interview that Dr. Braun's Declaration would substantiate these points. However, for reasons unknown to the Appellant, the Examiner stated in the Advisory Action (Exhibit 1) that she had not considered the Braun Declaration "... because Applicant has not shown good and sufficient reasons why it was not earlier presented..." (Advisory Action [Exhibit 1], page 2). It had seemed self-evident that presentation of this Declaration could not have preceded the clarification of issues achieved at the interveiw.

to unmodified cells, or establish that it achieved any clinical benefit (Braun Declaration [Exhibit 16], ¶¶ 9, 10, 11).

With these considerations in mind, we turn to the specific grounds for rejection.

a. The rejection of claims 47, 65-72 and 74-77 over Murphy, Berd 1989, Geczy, and the Antibody Patents

Claim 47 is directed to a method for treating a malignant tumor in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the patient, along with an adjuvant, at least six times. As disclosed in Examples 3, 4, and 6, administration of the immunotherapeutic vaccine comprising haptenized tumor cells on at least six, and in most cases eight, occasions resulted in actual treatment of tumors, with statistically significant greater cancer-free survival compared to controls (who received non-haptenized vaccine) at two years. (Specification, page 29, lines 22-25). The difference was highly significant. (*Id.*, page 30, lines 26-27; page 41, line 24 to page 42, line 8).

The Examiner states that Murphy teaches a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide (CY), and administering a therapeutically effective amount of

autologous, irradiated DNP-conjugated melanoma cells mixed with BCG adjuvant. The Examiner notes that Murphy does not teach administration of at least six vaccine doses at spaced intervals, a specific dose of CY (300 mg/M^2), prior sensitization with 1-fluoro-2,4-dinitrobenzene, or eliciting certain specified immune responses against the tumor. The Examiner cites the Antibody Patents for teaching administration of at least six doses of antigen; Berd 1989 for teaching a successful method of treating melanoma with the specified dosage of CY using DNP-conjugated melanoma cells, and that Geczy teaches that halogenated dinitrobenzenes are commonly used to elicit delayed type hypersensitivity. (Paper 36 [Exhibit 4], pages 9-10). The thrust of this rejection, then, is that it would have been obvious from the combined teachings of Murphy, the Antibody Patents, and Berd 1989 to administer at least six doses of a haptentized tumor cell vaccine, and that various haptenization reagents can be used as describe by Geczy.

Appellant have previously argued that the references cannot be combined as suggested by the Examiner without employing impermissible hindsight from the disclosure of the invention. The Examiner contends that "[s]ome degree of hindsight is permissible in making rejections under 35 U.S.C. 103 since it must be recognized that any judgement on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning." (Final Office Action [Exhibit 2], page 3).

The teachings and deficiencies of Geczy have been discussed in Appellant's previous amendment, filed September 22, 2000, on pages 11-12, paragraphs 3.b.iii. Geczy indeed teaches the equivalency of DNCB and DNFB for the induction of DTH as stated by the Examiner, but proposes that direct haptenization of lymphocytes is necessary for lymphocyte transformation. Thus, to the extent that the teachings of Geczy relate to those of Berd 1989 and/or Murphy, they diverge and teach away from using haptens to elicit a protective immune response against unmodified tumor cells. Geczy relates to anti-hapten responses, which would hardly be relevant to anti-tumor responses elicited by haptenized tumor cells, but only for immune response towards the haptenized tumor cell vaccine itself.

With respect to the Antibody Patents, their teachings and deficiencies were also discussed in the previous amendment dated September 22, 2000, pages 10-11, paragraph 3.b.ii. The Examiner states that the '704 patent, '843 patent, '183 patent, and '001 patent teach conventional immunization schedules wherein antigen is administered at least six times at spaced intervals. Indeed, the '183 patent teaches an assay for detecting the presence or absence of antibodies that bind to a human retrovirus antigen. The '001 patent teaches non-human antibodies to estrophilin. The '843 patent teaches the use of antibodies as immunosorbents for the treatment of AIDS. The '704 patent teaches antibodies that recognize advanced glycosylation endproducts and methods of using the antibodies for the measurement of the amount of advanced glycosylation end products in plants, animals, and cultivated and

synthesized protein material. None of these patents contains an objective teaching of the use of antibodies for the treatment of human cancers. Even if these patents did teach antibody-based (passive) immunotherapy, Appellant submits that such teaching would have no bearing on the present invention, which concerns active specific immunotherapy to tumors.

Moreover, the Antibody Patents fail to suggest, and indeed teach away from, generating an immune response to a carrier, such as a tumor cell, by immunizing with a hapten. Such a result would actually be contrary to the teachings of these references. For example, advanced glycosylation endproducts are analogous to haptens: they are small molecules conjugated to carrier proteins. Following the teaching of the '704 patent, one would expect to generate antibodies to the hapten. As pointed out above, such a result would be contrary to the invention, since an anti-hapten immune response would not affect residual tumor cells remaining after resection or metastasis.

As the Examiner has noted, these patents teach conventional methods for generating an antigen specific antibody response. Such antibodies are useful as diagnostic reagents. The immunized subjects do not develop protective immunity to the immunogen; that is not the intention or the outcome. Thus, one might conclude that by following the teachings of these patents, one would be unlikely to generate a protective immune response.

Furthermore, these references do not teach or suggest at least six administrations or immunizations of haptenized tumor cells (or any pathogenic antigen) to humans at spaced intervals for the treatment of cancer. No objective teaching thus exists in these patents that would suggest to or motivate one of skill in the art to administer antibodies to humans at least six times at spaced intervals in order to treat cancer.

The Examiner has made a number of legal errors to arrive at a conclusion of obviousness based on the combined teachings of these references, primarily by failing to properly articulate the Graham factors. For example, Examiner did not properly consider the scope and content of the prior art, and the differences between the prior art and the claimed invention. *Ruiz*, 57 USPQ2d at 1167. The Examiner further failed to establish the level of ordinary skill in the art, *Id.* at 1168, which Appellant has established through the Braun Declaration as well as through the references cited by the Examiner.

With respect to considering the scope and content of the prior art and the differences between the prior art and the claimed invention, the Examiner failed to articulate "... a reason, suggestion, or motivation in the prior art or elsewhere that would have led one of ordinary skill in the art to combine the references." *Id.* at 1167, *citing In re Rouffet*, 47 USPQ2d 1453, 1459 (Fed. Cir. 1998) and *In re Dembiczak*, 50 USPQ2d 1614, 1617 (Fed. Cir. 1999). The Federal Circuit provides explicit

guidance in *Ruiz* on the factual findings to make in determining a reason, suggestion, or motivation to combine:

> The reason, suggestion, or motivation to combine may be found explicitly or implicitly: 1) in the prior art references themselves; 2) in the knowledge of those of ordinary skill in the art that certain references, or disclosures in those references, are of special interest or importance in the field; or 3) from the nature of the problem to be solved, "leading inventors to look at references relating to possible solutions to that problem."

Id. (citations omitted). The Examiner merely alludes to a "conventional immunization scheduled" (Final Office Action [Exhibit 2], page 4; Paper 36 [Exhibit 4], page 11) without providing any basis for linking a conventional immunization schedule for eliciting diagnostic antibodies to an immunotherapy regimen.

The error here arises from the Examiner falling "into the hindsight trap." *In re Kotzab*, 55 USPQ2d 1313, 1318 (Fed. Cir. 2000). As the Court stated in *Kotzab*, "... to establish obviousness based on a combination of the elements disclosed in the prior art, there must be some motivation, suggestion or teaching of the desirability of making the specific combination that was made by the applicant." *Id.* at 1316, *citing In re Dance*, 48 USPQ2d 1635, 1637 (Fed. Cir. 1998); *In re Gordon*, 211 USPQ 1125, 1127 (Fed. Cir. 1984). The Antibody Patents teach methods to elicit antibodies to the immunizing agent, e.g., a hapten-like compound such as an advance glycosylation endproduct. Geczy shows that haptenization results in hapten-specific immunity. Neither Murphy nor Berd 1989 suggest at least six administrations of the immunotherapeutic vaccine, much less the advantages of doing so disclosed in

the Examples of the instant application. Thus, the Examiner has "... found prior art statements that in the abstract appeared to suggest the claimed limitation...", *Id.* at 1318, but which, in fact, lack any motivation to modify the teachings of Murphy or Berd 1989 to include that limitation.

With respect to the level of skill in the art, which the Examiner relies on in making this rejection (Final Office Action [Exhibit 2], page 4), Appellant respectfully submits that, in view of the Braun Declaration and the explicit teaching of the references, the level of skill in the art does not supply the missing teaching here. *See A-Site Corp. v. VSI*, 50 USPQ2d 1161, 1171 (Fed. Cir. 1999). "[T]he level of skill in the art is a prism or lens through with a judge or jury views the prior art and the claimed invention. This reference point prevents these deciders from using their own insight or, worse yet, hindsight, to gauge obviousness." *Id.* The Examiner has not established that the level of skill in the art is such that it would lead the skilled artisan to modify the teachings of Murphy or Berd 1989 as set forth in the claim. To rely on "conventional immunization schedules" is therefore error.

The Examiner cited two cases to support her analysis of obviousness. In particular, the Examiner points out that "[t]he test for obviousness...is what the combined teachings of the references would have suggested to those of ordinary skill in the art." *In re Keller*, 208 USPQ 871, 881 (CCPA 1981) (Citations omitted). Appellant agrees, but points out that the Examiner must consider the references for all that they teach; it is impermissible to consider a reference in less than its entirety,

or to disregard disclosures that diverge and teach away. *W.L. Gore & Assoc., Inc. v. Garlock, Inc.*, 220 USPQ 303 (Fed. Cir. 1993), *cert. denied* 469 U.S. 851 (1984). Such teaching away, which is the effect of the Antibody Patents and Geczy reference, defeats obviousness. *See Winner Int'l Royalty Corp. v. Wang*, 53 USPQ2d 1580, 1587 (Fed. Cir. 2000). Moreover, the prior art, and not the disclosure in the application, must both suggest the invention and provide a reasonable expectation of success in achieving it. *In re Vaeck*, 20 USPQ 2d 1438 (Fed. Cir. 1991).

Correctly applying the foregoing legal principles, the references neither (1) suggest the invention, nor (2) provide a reasonable expectation of success. Berd 1989 and by extension Murphy 1990 are inadequate to suggest modifying the immunization strategy to require at least six administrations of an immunotherapeutic vaccine. Taken together, as the Examiner points out that the references must be considered, the references do not render the claimed invention obvious. The Examiner's failure to properly consider the *Graham* factors, and the admitted use of hindsight to establish obviousness, represent error and should be reversed.

b. The rejection of claims 47, 65-72 and 74-77 over Berd 1989, the Antibody Patents, and Geczy

The Examiner maintained this rejection for the same reasons described in the above rejection. For the reasons set forth above, the combination of Berd 1989,

the Antibody Patents, and Geczy, fails to render the instant invention obvious. Indeed, this rejection is clearly ineffective since Berd 1989 provides even less information about methods of treatment and therapeutic outcomes than Murphy, which the Braun Declaration makes abundantly clear. In short, one of ordinary skill in the art would not have had any motivation to modify the teachings of Berd 1989 to require immunization with the haptenized tumor cell vaccine at least six times. The Examiner has erroneously failed to establish the *Graham* factors sufficient to render the invention obvious, and instead has substituted hindsight to sustain this rejection, both of which constitute error. *See Ruiz*, 57 USPQ2d at 1167-68. Thus, this rejection is in error and should be reversed.

c. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd 1989, the Antibody Patents, and Geczy in view of Wiseman

Claim 43 is directed to a composition comprising human tumor cells (other than melanoma cells) conjugated with a hapten. The haptenized tumor cells are obtained from the patient receiving treatment (i.e., they are "autologous"), and are rendered incapable of growing in the body of a human upon injection therein.

Claim 44 is directed to a method for treating a malignant tumor (other than melanoma) in a human patient by co-administering a composition comprising haptenized autologous human tumor cells of the same tumor type as the tumor in the

patient, along with an adjuvant. The composition elicits at least one of the following upon administration to the patient with the adjuvant: an inflammatory immune response against the tumor of the patient; a delayed-type hypersensitivity response against the tumor of the patient; and activated T lymphocytes that infiltrate the tumor of the patient.

Claim 47 has been discussed above, as have the references except for Wiseman. The Examiner contends that "Wiseman clearly showed that autologous irradiated melanoma, lung, colon, and kidney cancer were successfully used for successful immunological treatment of those cancers and it would have been expected that these cell types, already known in the art to be useful as immunogenic cancer treatments would be successfully substituted for the melanoma cells of Berd [1989] in order to treat the other cancer types." (Final Office Action [Exhibit 2], paragraph bridging pages 5 and 6).

The Braun Declaration addresses the teachings and deficiencies of Berd 1989, and discussed above. The reference lacks teachings with respect to any clinically significant tumor regression being observed, as well as the numbers and route of administration. (Braun Declaration [Exhibit 16], ¶¶9 and 11). Thus, one of ordinary skill in the art would have presumed that Berd 1989's haptenized tumor cells and BCG had been injected intratumorally, and that the BCG was thereby responsible for the observed, clinically non-significant, tumor responses (Braun Declaration [Exhibit 16],¶11). Accordingly, Berd 1989 suffers from a lack of expectation of success for

the haptenized-tumor-cell approach in melanoma, and even more so in the case of non-melanoma tumors.

As discussed in the previous amendment dated September 22, 2000 (p. 17, 1st full paragraph), Wiseman teaches an alternative form of immunotherapy that depends on the route of administration: intralymphatic immunization. This alternative, which Wiseman indeed reports favorably, in no way suggests a deficiency or problem that would lead one of ordinary skill in the art to seek an alternative immunization strategy.

On the contrary, Wiseman diverts the skilled artisan away from the claimed invention, thus precluding combining this reference in making the rejection. One of ordinary skill in the art would, when provided with the Wiseman reference on one hand and the combination of Berd 1989, The Antibody Patents, and Geczy on the other, the latter themselves leading away from any combination with the haptenized tumor cell compositions and methods because they suggest an anti-hapten rather than anti-tumor cell response, be inclined to pursue the Wiseman approach: intralymphatic immunization with unmodified tumor cells, since this approach was successful and avoided the problems with the haptenization approach in Berd 1989 and suggested by the Antibody Patents and Geczy. Such teaching away, which is the effect of the Antibody Patents and Geczy reference, defeats obviousness. *See Winner Int'l Royalty*, 53 USPQ2d at 1587.

Nothing in Berd 1989 suggests that haptenization of tumor cells provides an effective therapeutic response (as established by the Braun Declaration), much less a more effective response than other immunization protocols. However, the data clearly support the unexpected superiority of the haptenized tumor cells and methods of immunotherapy using them.

> The DFS [disease-free survival] and TS [total survival] of [patients treated with haptenized tumor cells] were compared with those of 22 melanoma patients with resected nodal metastasises treated previously with unconjugated vaccine, see Example 4. Of 36 patients with stage 3 melanoma (palpable mass in one lymph node region), 22 are disease-free with a median follow-up of 33 months. Kaplan-Meir analysis projects a 3 year DFS and TS of 59% and 71%, respectively. In contrast, the DFS and TS of stage 3 patients treated with unconjugated vaccine was 22% and 27% respectively (p = 0.01, log-rank test). Of 11 stage 4 patients (palpable mass in two lymph node regions), 5 are NED (no evidence of disease) with a median follow-up of 41 months.

(Specification, page 41, line 24 to page 42, line 8). These data demonstrate the superiority of the claimed invention, particularly the claimed methods of treatment, relative to Wiseman's approach. However, these advantages can only be gleaned from the disclosure of the specification, and are not available from the combined teachings of the references. Advantages flowing directly from the invention are one consideration that may be relevant to a determination of obviousness. *Mosinee Paper Corp. v. James River Corp. of Virginia*, 22 U.S.P.Q.2d 1657, 1660, *aff'd. mem.* 980 F.2d 743 (Fed. Cir. 1992) (citing *Pre-Emption Devices, Inc. v. Minnesota Mining & Mfg. Co.*, 221 USPQ 841 (Fed. Cir. 1984)). "After all, those advantages are the foundation of that 'commercial success' which may be evidence of nonobviousness.

Pre-Emption, supra, at 844 (*citing Graham*, 383 U.S. at 17). Thus, the showing of significant advantages of the presently claimed compositions and methods, particularly as related in Example 6 (quoted above), demonstrates non-obviousness of the invention.

Even if Berd 1989 taught an effective immunotherapy of melanoma using haptenized, autologous melanoma cells, such a teaching would not form a sufficient basis for combination with Wiseman to achieve the claimed invention. As applicants have previously pointed out, it is not expected that "vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al." (See Paper No. 41 [Exhibit 3], page 5, lines 12-14). In the PTO-1449 form filed by Applicant on December 1, 1998, Applicant brought the Examiner's attention to Hanna et al. (U.S. Patent No. 5,484,596, hereinafter "Hanna"). Hanna teaches a method for the treatment of human colon cancer that involves the use of a vaccine which is made from irradiated human tumor cells. The Examiner is requested to note that the Hanna et al. vaccine strategy appears to be effective only for treating colon cancer. A publication reporting on a clinical trial of the "Hanna et al." vaccine concedes that the vaccine was not effective for rectal cancer (Hoover et al., J. Clin. Oncology 11: 390-399, 1993; copy attached to the Amendment filed September 22, 2000 as Exhibit 1 [Exhibit 17]). Hoover et al. states that ". . . no benefits were seen in patients with rectal cancer who received [active specific immunotherapy with an autologous

tumor cell-BCG vaccine]" (see Abstract; see also page 399, first column). Hence, although the Hanna vaccine was reportedly successful in treating colon cancer, it failed to provide any benefits to patients with rectal cancer, a tumor type closely related to colon cancer. Accordingly, even had Berd '89 successfully treated melanoma patients with his haptenized tumor cell vaccine, and not only provided preliminary and essentially anecdotal results relating to DTH-responses, it could not have been reasonably expected that a similar vaccine would be equally effective in the treatment of related tumors, much less tumors of completely unrelated origin.

It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, here to the Examiner's citations appear on the surface to suggest the claimed invention, but, upon further review, can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course, improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

d. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd 1989, the Antibody Patents, Geczy, and Berd 1983

Claims 43, 44, and 47 have been discussed above. Berd, the Antibody Patents, and Geczy are discussed above. In addition, the teachings of Berd 1989, the

Antibody Patents, and Geczy have been discussed above. The Examiner states that Berd '83 teaches treatment of breast cancer patients with autologous vaccine, and that the substitution of the breast cancer cells of Berd 1983 for the melanoma cells of Berd 1989 was prima facie obvious.

The Examiner alleges that Appellant has previously argued the Berd 1983 reference individually without clearly addressing the combined teachings. Appellant respectfully disagrees. Appellant chose to, instead of repeating arguments already made in the amendment, discuss the entirety of the teachings of Berd 1983 before adding this reference to the combination of Berd 1989, the Antibody Patents, and Geczy (see amendment dated September 22, 2000, page 18, section 3.g). In doing so, it is clear that Berd 1983 adds nothing to the combination of Berd 1989, The Antibody Patents, and Geczy, which combination is (1) improper and (2) fails to provide any reasonable expectation of success as discussed above.

Berd 1983 teaches the intradermal administration of autologous tumor cells to six cancer patients, five suffering from melanoma and one from breast cancer, and reports DTH responses against tumor cells in three out of the five evaluated patients. Note that Berd 1983 is silent with respect to whether the single breast cancer patient was among the 3 patients (50%) showing a DTH response. Even assuming that the breast cancer patient was among the three, the addition of Berd 1983 to the combination of reference would not provide a reasonable expectation that

a haptenized tumor cell vaccine, whether based on melanoma or breast cancer cells, would elicit a clinically significant anti-tumor response.

The Braun Declaration addresses the teachings and deficiencies of Berd 1989, as discussed above. The reference lacks teachings with respect to any clinically significant tumor regression being observed, as well as the numbers and route of administration. (Braun Declaration [Exhibit 16], ¶¶9 and 11). Thus, one of ordinary skill in the art would have presumed that Berd 1989's haptenized tumor cells and BCG had been injected intratumorally, and that the BCG was thereby responsible for the observed, clinically non-significant, tumor responses (Braun Declaration [Exhibit 16],¶11). Accordingly, Berd 1989 suffers from a lack of expectation of success for the haptenized-tumor-cell approach in melanoma, and even more so in the case of non-melanoma tumors.

As noted above, even if Berd 1989 taught an effective immunotherapy of melanoma using haptenized, autologous melanoma cells, such a teaching would not form a sufficient basis for combination with Berd 1983 to achieve the claimed invention. As applicants have previously pointed out, it is not expected that "vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al." (See Paper No. 41 [Exhibit 3], page 5, lines 12-14). In the PTO-1449 form filed by Applicant on December 1, 1998, Applicant brought the Examiner's attention to Hanna et al. (U.S. Patent No. 5,484,596, hereinafter "Hanna"). Hanna teaches a method for

the treatment of human colon cancer that involves the use of a vaccine which is made from irradiated human tumor cells. The Examiner is requested to note that the Hanna et al. vaccine strategy appears to be effective only for treating colon cancer. A publication reporting on a clinical trial of the "Hanna et al." vaccine concedes that the vaccine was not effective for rectal cancer (Hoover [Exhibit 17]). Hoover et al. states that ". . . no benefits were seen in patients with rectal cancer who received [active specific immunotherapy with an autologous tumor cell-BCG vaccine]" (see Abstract; see also page 399, first column). Hence, although the Hanna vaccine was reportedly successful in treating colon cancer, it failed to provide any benefits to patients with rectal cancer, a tumor type closely related to colon cancer. Accordingly, even had Berd '89 successfully treated melanoma patients with his haptenized tumor cell vaccine, and not only provided preliminary and essentially anecdotal results relating to DTH-responses, it could not have been reasonably expected that a similar vaccine would be equally effective in the treatment of related tumors, much less tumors of completely unrelated origin.

It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, here to the Examiner's citations appear on the surface to suggest the claimed invention, but, upon further review, can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course,

improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

e. The rejection of claims 43, 44, 47, 49-62, 64-72, and 74-77 over Berd, the Antibody Patents, Geczy, and Sanda and Moody

Claims 43, 44, and 47 have been discussed above. Berd, the Antibody Patents, and Geczy are discussed above. In addition, the teachings of Berd 1989, the Antibody Patents, and Geczy have been discussed above. The Examiner has stated that in particular Berd supplies the motivation to "decorate the tumor cells with hapten." Incorporating the reasoning set forth in the prior two Office Actions, the Examiner states that Moody teaches that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo*, and that Sanda addresses the feasibility of gene therapy for human prostate cancer. These references appear to be relevant to the Examiner because they suggest methods of anti-prostate cancer therapy.

Sanda teaches a method for transducing human prostate cancer cells with a particular retroviral vector. The method was reportedly successful for transfecting the cells, and Sanda suggest that this approach may be feasible in gene therapy of prostate cancer. Although Sanda fails to provide any description on just how his approach would be used in gene therapy and why, the general approach in gene therapy is to administer a gene, locally or systemically, to a patient in an attempt to

transfect cells *in vivo* for some therapeutic purpose. This approach is wholly unrelated to any immunotherapeutic method for cancer treatment, and therefore cannot add anything to the combination of references cited in this rejection. It solves a different problem (tumor cell ablation through a therapeutic gene) than the claimed invention (tumor cell immunotherapy using a haptenized tumor cell as a vaccine). Thus, there is no logical connection between Sanda and the other references cited in this rejection. *See Ruiz*, 57 USPQ2d at 1168 (evidence that the references solve different problems can preclude a determination of obviousness).

Moody teaches an immunotherapy method based on lymphokine expression in which rat prostate tumor cells were transfected with cDNA encoding IL-2 and IL-4, the transfected tumor cells were administered to rats, and tumor immunity observed in the treated rats as compared to controls. This references adopts an altogether different approach than haptenization of tumor cells to elicit tumor cell immunity.[5] There is no suggestion from Moody to modify the approach it teaches by haptenizing the prostate tumor cells.

In short, for the reasons discussed above, the combination of Berd 1989, the Antibody Patents, and Geczy fail to suggest, much less teach, compositions of haptenized tumor cells (that are not melanoma cells), methods of cancer

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[5] As disclosed in the specification of the instant application, using immunostimulatory molecules in <u>combination</u> with the claimed compositions and methods may be desirable in some instances. (Specification, page 17, lines 17-20). Moody provides one avenue for such a combination. This in no way suggests the claimed invention.

immunotherapy using haptenized tumor cells (especially that are not melanoma cells as recited in claim 44), or methods of cancer immunotherapy involving a regimen of administering haptenized tumor cells at least six times. Sanda and Moody have nothing to do with immunotherapy; they are in this respect less relevant than Wiseman. Accordingly, for the reasons advanced above, this rejection is in error and must be reversed.

It is clear that upon careful examination, the references cannot be combined as the Examiner has suggested. Thus, here to the Examiner's citations appear on the surface to suggest the claimed invention, but, upon further review, can only be combined as the Examiner proposes with knowledge of the Applicant's invention. *In re Kotzab*, 55 USPQ2d at 1318. Such an analysis is, of course, improper. For the foregoing reasons, this obviousness rejection is in error and should be reversed.

9. Conclusion

For the forgoing reasons, Appellant submits that the Final Rejection is in error and should be reversed on all grounds. The Examiner has committed error by failing across the board to properly articulate the *Graham* factors. For example, Examiner did not properly consider the scope and content of the prior art, and the differences between the prior art and the claimed invention. *Ruiz*, 57 USPQ2d at

1167. The Examiner further failed to establish the level of ordinary skill in the art, *Id.* at 1168, which Appellant has established through the Braun Declaration as well as through the references. With respect to considering the scope and content of the prior art and the differences between the prior art and the claimed invention, the Examiner failed to articulate "... a reason, suggestion, or motivation in the prior art or elsewhere that would have led one of ordinary skill in the art to combine the references." *Id.* at 1167 (citations omitted). Instead, the Examiner has improperly relied on the level of skill in the art to fill in the holes in the prior art, *See A-Site Corp.* 50 USPQ2d at 1171, and has consistently fallen into the "hindsight trap". *In re Kotzab*, 55 USPQ2d at 1318.

Respectfully submitted,

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APPENDIX

Pending Claims on Appeal

43. (Amended) A composition comprising human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein;

said composition eliciting, when administered together with an adjuvant, an inflammatory immune response against the tumor of said patient, wherein said tumor is not melanoma.

44. A method for treating a malignant tumor in a human patient comprising co-administering to the patient

(a) a composition comprising a therapeutically effective amount of human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein; and

(b) an adjuvant;

wherein said composition elicits at least one of the following upon administration to said patient with the adjuvant: an inflammatory immune response against the tumor of said patient; a delayed-type hypersensitivity response against the

tumor of said patient, and activated T lymphocytes that infiltrate the tumor of said patient, wherein said malignant tumor is not melanoma.

47. (Amended) A method of treating a malignant tumor in a human patient comprising co-administering to the patient

(a) a composition comprising a therapeutically effective amount of human tumor cells that:

(i) are conjugated to a hapten;

(ii) are of the same tumor type as a malignant tumor of a patient for treatment of whom the composition is intended;

(iii) are autologous to said patient; and

(iv) have been rendered incapable of growing in the body of a human upon injection therein; and

(b) an adjuvant;

wherein said composition elicits at least one of the following upon administration to said patient with the adjuvant: an inflammatory immune response against the tumor of said patient; a delayed-type hypersensitivity response against the tumor of said patient and activated T lymphocytes that infiltrate the tumor of said patient; and

repeating said administration at least six times at spaced apart intervals.

49. The composition of claim 43 wherein said tumor cells are selected from lung, colon, breast, kidney, and prostate tumor cells.

50. The composition of claim 43 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5 sylfonic 1-naphtyl) ethylene diamine.

51. The composition of claim 43 wherein said hapten is dinitrophenyl.

52. The composition of claim 43 further comprising an adjuvant.

53. The composition of claim 52 wherein said adjuvant is Bacillus Calmette-Guerin.

54. The composition of claim 43 further comprising a carrier.

55. The composition of claim 54 wherein said carrier is selected from the group consisting of saline solution and culture medium.

56. The method of claim 44 wherein said tumor cells are selected from lung, colon, breast, kidney, and prostate tumor cells.

57. The method of claim 44, wherein said malignant tumor is from a cancer selected from the group consisting of lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

58. The method of claim 44 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

59. The method of claim 44 wherein said hapten is dinitrophenyl.

60. The method of claim 44 further comprising administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition.

61. The method of claim 60 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M2 of cyclophosphamide prior to administration of said composition.

62. The method of claim 60 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

64. The method of claim 44 wherein said adjuvant is Bacillus Calmette-Guerin.

65. The method of claim 47 wherein said tumor cells are selected from melanoma, lung, colon, breast, kidney, and prostate tumor cells.

66. The method of claim 47, wherein said malignant tumor is from a cancer selected from the group consisting of melanoma cancer, lung cancer, colon cancer, breast cancer, kidney cancer, and prostate cancer.

67. The method of claim 47 wherein said hapten is selected from the group consisting of dinitrophenyl, trinitrophenyl, and N-iodoacetyl-N'-(5-sulfonic 1-naphtyl) ethylene diamine.

68. The method of claim 47 wherein said hapten is dinitrophenyl.

69. The method of claim 47 further comprising administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition.

70. The method of claim 47, further comprising administering a therapeutically effective amount of cyclophosphamide prior to the first administration of said composition.

71. The method of claim 69 wherein said therapeutically effective amount of cyclophosphamide comprises administering a dose of about 300 mg/M2 of cyclophosphamide prior to administration of said composition.

72. The method of claim 47 further comprising sensitizing the patient with a therapeutically effective amount of 1-fluoro-2,4-dinitrobenzene prior to administering cyclophosphamide.

74. The method of claim 47 wherein said adjuvant is Bacillus Calmette-Guerin.

75. The method of claim 47 wherein said administration prolongs survival of said patient.

76. The method of claim 44, wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8 + CD4 .

77. The method of claim 47, wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8 + CD4 .

Exhibit 1

SERIAL NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/203,004	02/28/94	BERD	1225/0C674

08/203,004

Brief

DUE: July 29 2001

Docketed on 7/10 by DP for

Docketed without file ☐

Attorney

EXAMINER	
UNGAR, S	
ART UNIT	PAPER NUMBER
1642	48

DATE MAILED: 07/05/01

12/29/01

DARBY & DARBY P.C.
805 THIRD AVENUE
NEW YORK NY 10022

Below is a communication from the EXAMINER in charge of this application

COMMISSIONER OF PATENTS AND TRADEMARKS

ADVISORY ACTION

☑ THE PERIOD FOR RESPONSE:

a) ☐ is extended to run _____ or continues to run _____ from the date of the final rejection

b) ☐ expires three months from the date of the final rejection or as of the mailing date of this Advisory Action, whichever is later. In no event however, will the statutory period for the response expire later than six months from the date of the final rejection.

Any extension of time must be obtained by filing a petition under 37 CFR 1.136(a), the proposed response and the appropriate fee. The date on which the response, the petition , and the fee have been filed is the date of the response and also the date for the purposes of determining the period of extension and the corresponding amount of the fee. Any extension fee pursuant to 37 CFR 1.17 will be calculated from the date of the originally set shortened statutory period for response or as set forth in b) above.

☑ Appellant's Brief is due in accordance with 37 CFR 1.192(a).

☐ Applicant's response to the final rejection, filed June 2, 2001 has been considered with the following effect, but it is not deemed to place the application in condition for allowance:

1. ☐ The proposed amendments to the claim and /or specification will not be entered and the final rejection stands because:

   a. ☐ There is no convincing showing under 37 CFR 1.116(b) why the proposed amendment is necessary and was not earlier presented.

   b. ☐ They raise new issues that would require further consideration and/or search. (See Note).

   c. ☐ They raise the issue of new matter. (See Note).

   d. ☐ They are not deemed to place the application in better form for appeal by materially reducing or simplifying the issues for appeal.

   e. ☐ They present additional claims without cancelling a corresponding number of finally rejected claims.

NOTE: _____

2. ☐ Newly proposed or amended claims _____ would be allowed if submitted in a separately filed amendment cancelling the non-allowable claims.

3. ☑ ~~Upon the filing an appeal, the proposed amendment~~ The AMENDMENT has been entered ☐ ~~will be entered~~ ☐ ~~will not be entered~~ and the status of the claims will be as follows:

Claims allowed: None

Claims objected to: None

Claims rejected: 43, 44, 47, 49-62, 64-72, 74-77

However;

☑ Applicant's response has overcome the following rejection(s): Rejection of Cl 43, 49-51, 54-55 under 112 1st para

4. ☐ The affidavit, exhibit or request for reconsideration has been considered but does not overcome the rejection because _____

5. ☑ The affidavit or exhibit will not be considered because applicant has not shown good and sufficent reasons why it was not earlier presented.

☐ The proposed drawing correction ☐ has ☐ has not been approved by the examiner.

☐ Other

Susan Ungar

BEST AVAILABLE COPY

1. The Amendment After-Final filed June 2, 2001 (Paper No. 46) in response to the Office Action of November 29, 2000 (Paper No. 44) is acknowledged and has been entered. Previously pending claims 43 and 47 have been amended. Claims 43, 44, 47, 49-62, 64-72 and 74-77 are currently being examined.

Claim Rejections - 35 USC § 103

2. Claims 47, 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 10-11, pages 8-15, Paper No. 41, Sections 5-6, pages 2-4 and in Paper No. 44, Sections 5 and 6, pages 3-5.

Applicant argues that (a) Examiner gives more weight to the Berd '89 abstract than one of ordinary skill at the time the invention would have given it and the Braun Declaration sets forth that Berd '89 does not describe a successful immunotherapy for melanoma, (b) Applicant details the three basic criteria that must be met to establish a *prima facie* case for obviousness under 35 USC 103 and states that since Berd '89 fails to provide any expectation of success the reference is completely irrelevant and no other reference provided makes up for this flaw, (c) Applicant reiterates the deficiencies of Murphy, Berd '89 Geczy and Antibody Patents and the combination thereof. The arguments have been considered but have not been found persuasive (a') for the reasons previously set forth in Paper No. 41, Section 7, pages 4-5. The Braun Declaration has not been considered because Applicant has not shown good and sufficient reasons why it was not earlier presented, (b') the Berd '89 reference is not irrelevant for the reasons set forth previously and above, (c') the arguments are not persuasive for the reasons previously set forth

3. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 12, pages 15-18, Paper No. 41, Section 7, pages 4-5 and in Paper No. 44, Section 7, pages 5-6.

Applicant reiterates the arguments drawn to Wiseman and the arguments drawn to the combination of Berd '89, The Antibody Patents and Geczy. The arguments have been considered but have not been found persuasive for the reasons previously set forth.

4. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 13, pages 18-21, Paper No. 41, Section 8, page 6 and in Paper No. 44, Section 8, page 6.

Applicant reiterates arguments drawn to Berd '83. The arguments have been considered but have not been found persuasive for the reasons previously set forth in Paper Nos 36, 41 and 44.

5. Claims 43, 44, 47, 49-62, 64-72, and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 14, pages 21-25, Paper No. 41, Section 9, page 7 and in Paper No. 44, Section 9, page 7.

Applicant reiterates the arguments previously set forth. The arguments have been considered but have not been found persuasive for the reasons previously set forth in Paper Nos 36, 41 and 44.

6. All other objections and rejections recited in Paper No. 44 are withdrawn.

SUSAN UNGAR, PH.D
PRIMARY EXAMINER

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NO.
08/203004			

EXAMINER	
ART UNIT	PAPER NUMBER
1642	

DATE MAILED:

INTERVIEW SUMMARY

All participants (applicant, applicant's representative, PTO personnel):

(1) S. UNGAR (3) _____

(2) P. Fahlvog (4) _____

Date of Interview 6/13/01

Type: ☒ Telephonic ☐ Personal (copy is given to ☐ applicant ☐ applicant's representative).

Exhibit shown or demonstration conducted: ☐ Yes ☐ No If yes, brief description: _____

Agreement ☐ was reached. ☐ was not reached.

Claim(s) discussed: All pending

Identification of prior art discussed: _____

Description of the general nature of what was agreed to if an agreement was reached, or any other comments:
Rec'd AF + Dec reviewing for patentable Material
will be back to AU w/ a few days

(A fuller description, if necessary, and a copy of the amendments, if available, which the examiner agreed would render the claims allowable must be attached. Also, where no copy of the amendments which would render the claims allowable is available, a summary thereof must be attached.)

1. ☐ It is not necessary for applicant to provide a separate record of the substance of the interview.

Unless the paragraph above has been checked to indicate to the contrary. A FORMAL WRITTEN RESPONSE TO THE LAST OFFICE ACTION IS NOT WAIVED AND MUST INCLUDE THE SUBSTANCE OF THE INTERVIEW. (See MPEP Section 713.04). If a response to the last Office action has are ready been filed, APPLICANT IS GIVEN ONE MONTH FROM THIS INTERVIEW DATE TO FILE A STATEMENT OF THE SUBSTANCE OF THE INTERVIEW.

2. ☐ Since the Examiner's interview summary above (including any attachments) reflects a complete response to each of the objections, rejections and requirements that may be present in the last Office action, and since the claims are now allowable, this completed form is considered to fulfill the response requirements of the last Office action. Applicant is not relieved from providing a separate record of the interview unless box 1 above is also checked.

Examiner Note: You must sign this form unless it is an attachment to another form.

FORM PTOL-413 (REV.1-96)

Attachment for PTO-948 (Rev. 03/01, or earlier)
6/18/01

The below text replaces the pre-printed text under the heading, "Information on How to Effect Drawing Changes," on the back of the PTO-948 (Rev. 03/01, or earlier) form.

INFORMATION ON HOW TO EFFECT DRAWING CHANGES

1. Correction of Informalities -- 37 CFR 1.85

New corrected drawings must be filed with the changes incorporated therein. Identifying indicia, if provided, should include the title of the invention, inventor's name, and application number, or docket number (if any) if an application number has not been assigned to the application. If this information is provided, it must be placed on the front of each sheet and centered within the top margin. If corrected drawings are required in a Notice of Allowability (PTOL-37), the new drawings **MUST** be filed within the **THREE MONTH** shortened statutory period set for reply in the Notice of Allowability. Extensions of time may NOT be obtained under the provisions of 37 CFR 1.136(a) or (b) for filing the corrected drawings after the mailing of a Notice of Allowability. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

2. Corrections other than Informalities Noted by Draftsperson on form PTO-948.

All changes to the drawings, other than informalities noted by the Draftsperson, **MUST** be made in the same manner as above except that, normally, a highlighted (preferably red ink) sketch of the changes to be incorporated into the new drawings **MUST** be approved by the examiner before the application will be allowed. No changes will be permitted to be made, other than correction of informalities, unless the examiner has approved the proposed changes.

Timing of Corrections

Applicant is required to submit the drawing corrections within the time period set in the attached Office communication. See 37 CFR 1.85(a).

Failure to take corrective action within the set period will result in **ABANDONMENT** of the application.

Exhibit 2

AL

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

VB

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/203,004	02/28/94	BERD	D 1225/0C674

EXAMINER	
UNGAR, S	
ART UNIT	**PAPER NUMBER**
1642	44

HM22/1129

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

FINAL

DUE: February 28, 2001

Docketed on 12/6 by DA for

Docketed without file ☐

Attorney

DATE MAILED: 11/29/00

5-29-01

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

2000 DEC -2 A 10:01

RECEIVED

PTO-90C (Rev. 2/95)

2 - Mail Copy

Office Action Summary	Application No. 08/203,004	Applicant(s) Berd
	Examiner Ungar	Group Art Unit 1642

☒ Responsive to communication(s) filed on *Sep 27, 2000* .

☒ This action is **FINAL.**

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *three* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *43, 44, 47, 49-62, 64-72, and 74-77* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *43, 44, 47, 49-62, 64-72, and 74-77* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____ .

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____ .

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. The Amendment filed September 27, 2000 (Paper No. 43) in response to the Office Action of March 22, 2000 (Paper No. 41) is acknowledged and has been entered. Previously pending claims 54, 55, 57, 66 and 70 have been amended. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 are currently being examined.

2. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

3. The following rejections are being maintained:

Claim Rejections - 35 USC § 112

4. Claims 43, 49-51, 54-55 remain rejected under 35 USC 112, first paragraph for the reasons previously set forth in Paper No. 41, Section 10, pages 7-8.

Applicant argues that the composition is a useful component of a vaccine and that the specification enables a haptenized tumor cell composition in combination with an adjuvant and that for that reason both components must be enabled. Applicant further cites case law to demonstrate that products are useful if they serve as starting materials in producing other materials which are directly useful and therefore Examiner errs in interpreting these claims as requiring adjuvant. The argument has been considered but has not been found persuasive because the claims are not drawn to a component of a vaccine that upon addition of an adjuvant will elicit an immune response but rather specifically recite the limitation that upon injection the composition (without adjuvant) elicits an inflammatory immune response. For the reasons previously set forth, without an adjuvant the claim is not enabled. Applicant's arguments have not been found persuasive and the rejection is maintained.

Claim Rejections - 35 USC § 103

5. Claims 47 and 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 10, pages 8-12 and in Paper No. 41, Section 5, pages 2 and 3.

Applicant argues (a) in order for a combination of prior art references to suggest a claimed invention, an objective teaching must exist in the prior art that would lead a skilled artisan to combine its teaching; (b) it is impermissible to use hindsight reconstruction to pick and choose among isolated disclosures in the prior art to deprecated the claimed invention, (c) Applicant takes issue with Examiner's contention that Applicant has argued and discussed the references individually without clearly addressing the combined teachings since each reference must be considered in its sentirely and then evaluation must be made as to whether the reference can be properly combined with the others and whether the combination suggests the claimed invention with a reasonable expecation of success (d) Applicant discusses the individual references, (e) Applicant reiterates the arguments drawn to the rejection of claims 47 and 65-76 in Paper No. 36, Section 10, pages 8-12.

The arguments have been considered but have not been found persuasive (a') for the reasons previously set forth in Paper No. 51 on page 3, it is suggested that Applicant review In re Keller, 642 F.2d 413, 208 USPQ 871 (CCPA 1981), (b') Some degree of hindsight is permissible in making rejections under 35 USC 103 since it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But, so long as it takes

into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. In re McLaughlin , 443 F.2d 1392, 170 USPQ 209 (CCPA 1971). It is clear that a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide and administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells mixed with BCG was well known in the art as taught by Murphy et al and that immunization schedules wherein antigen is administered on a schedule of at least six times at spaced intervals was conventional in the art as taught by the Antibody Patents cited on Page 10 of Paper No. 36 and that a dose of 300 mg/M^2 was successfully administered prior to autologous irradiated, DNP-conjugated melanoma cells as taught by Berd et al and the equivalence of halogenated dinitrobenzenes for the elicitation of delayed hypersensitivity was known in the art as taught by Geczy et al. Given the information known in the art, the combined references teach not only the suggestion but also the means and motivation to successfully treat a malignant tumor in a human patient as claimed, (c') upon review and reconsideration it is found that, in Examiner's view, Applicant did not clearly address the combined teachings of the references, (d') and (e') Applicant's arguments are not persuasive for the reasons previously set forth. Applicant's arguments have not been found persuasive and the rejection is maintained.

6. Claims 47 and 65-72 and 74-77 remain rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Section 11, pages 12-15 and in Paper No. 41, Section 6, page 4.

Applicant argues that for the reasons set forth above the rejection is in error and should be withdrawn. The argument has been considered but is not found persuasive for the reasons set forth above. Applicant's arguments have not been found persuasive and the rejection is maintained.

7. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 12, pages 15-18 and in Paper No. 41, Section 7, pages 4-5.

Applcant argues that (a) it is not expected that vaccines using other types of tumor cells would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al since Hanna et al teaches a method for treatment of human colon cancer using a vaccine made from irradiated human tumor cells and Hoover teaches that when comparing vaccines made from irradiated autologous cells of colon cancer patients and rectal cancer patients, only the colon cancer patients received benefit from the vaccine, (b) Applicant again argues that Wiseman does not supply the missing teaching and the "preliminary" nature of the results of Berd et al.

The arguments have been considered but have not been found persuasive because (a') Wiseman clearly showed that autologous irradiated melanoma, lung, colon and kidney cancer cells were successfully used for successful immunological treatment of those cancers and it would have been expected that these cell types,

already known in the art to be useful as immunogenic cancer treatments would be successfully substituted for the melanoma cells of Berd et al in order to treat the other cancer types. The fact that a single cancer cell type is not immunogenic is not relevant in view of the demonstrated efficacy of the cell types of Wiseman, (b') the arguments are not persuasive for the reasons previously set forth.Applicant's arguments have not been found persuasive and the rejection is maintained.

8. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 13, pages 18-21 and in Paper No. 41, Section 8, page 6.

Applicant argues that Berd '83 does not provide an incentive to prepare a haptenized tumor cell vaccine or methods of treating cancer using said vaccine. The argument has been noted but has not been found persuasive for the reasons previously set forth. Applicant is arguing this reference individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon refefrences which made up the state of teh art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981). Applicant's arguments have not been found persuasive and the rejection is maintained.

9. Claims 43, 44, 47 and 49-62, 64-72 and 74-77 remain rejected for the reasons previously set forth in Paper No. 36, Section 14, pages 21-25 and in Paper No. 41, Section 9, page 7.

Applicant reiterates the arguments previously set forth. The arguments have been considered but have not been found persuasive for the reasons previously set forth. Applicant's arguments have not been found persuasive and the rejection is maintained.

10. All other objections and rejections recited in Paper No. 41 are withdrawn.

11. No claims allowed.

12 **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a).

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is

(703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anthony Caputa, can be reached at (703) 308-3995. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642.

Susan Ungar
Primary Patent Examiner
November 28, 2000

Exhibit 3

PFF

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
Address: COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

VB

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.
08/203,004	02/28/94	BERD D	1225/0C674

EXAMINER	
UNGAR, S	
ART UNIT	**PAPER NUMBER**
1642	41

HM12/0322

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

DUE: June 22, 2000
Docketed on 3/27 by P P for
Docketed without file ☐
Attorney PFF

DATE MAILED: 03/22/00
9-22-00

Please find below and/or attached an Office communication concerning this application or proceeding.

Commissioner of Patents and Trademarks

Office Action Summary	Application No. 08/203,004	Applicant(s) Berd
	Examiner Ungar	Group Art Unit 1642

☒ Responsive to communication(s) filed on *Jan 14, 2000*.

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire *three* month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) *43, 44, 47, and 49-77* is/are pending in the application.

Of the above, claim(s) _____ is/are withdrawn from consideration.

☐ Claim(s) _____ is/are allowed.

☒ Claim(s) *43, 44, 47, and 49-77* is/are rejected.

☐ Claim(s) _____ is/are objected to.

☐ Claims _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.

☐ The drawing(s) filed on _____ is/are objected to by the Examiner.

☐ The proposed drawing correction, filed on _____ is ☐approved ☐disapproved.

☐ The specification is objected to by the Examiner.

☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.

☐ received in Application No. (Series Code/Serial Number) _____.

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Interview Summary, PTO-413

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

--- SEE OFFICE ACTION ON THE FOLLOWING PAGES ---

1. The Amendment filed November 4, 1999 (Paper No. 38) in response to the Office Action of April 28, 1999 (Paper No. 36) is acknowledged and has been entered. The Amendment (Paper No. 39) and the Terminal Disclaimer (Paper No. 40) filed January 14, 2000, in response to the telephone interview of January 13, 2000 (Paper No. 38.5) have been entered Previously pending claims 63 and 73 have been canceled, claims 44, 47, 64, 74 and 76 have been amended and new claim 77 has been added. Claims 43, 44, 47 49-62, 64-72 and 74-77 are currently being examined.

2. The Terminal Disclaimer filed January 14, 2000 is acceptable.

3. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

4. The following rejections are being maintained:

Claim Rejections - 35 USC § 103

5. Claims 47 and 65-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 10 , pages 8-12.

Applicant argues that (a) the legal test for obviousness cannot be established by combining teachings of the prior art absent some teaching or suggestion supporting the combination. Under section 103, teachings of references can be combined only if there is some suggestion or incentive to do so, (b) Murphy and or Berd fail to teach a method of treatment for non-melanoma malignant tumors in a human patient, (c) the antibody patents which merely teach conventional methods for generating antibodies provide no missing teaching, (d) Geczy fails to provide any teaching pertinent to the claimed compositions and methods.

The arguments have been considered but have not been found persuasive because (a') contrary to the assertion of Applicant, the test for obviousness is not that the claimed invention must be expressly suggested in any one or all of the references; but rather the test is what the combined teachings of the references would have suggested to those of ordinary skill in the art, (b') Applicant is arguing limitations not recited in the claims as currently constituted. The claims are drawn to a method of treating a malignant tumor in a human patient and as broadly written, read on the treatment of melanoma (b'-d') Applicant has argued and discussed the references individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which made up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981), (c') multiple immunizations are conventional in the art for producing all forms of immune response, (d') Geczy clearly teach the equivalence of CDNB and FDNB. The references teach not only the suggestion but also the means and motivation to successfully treat melanoma by sensitizing with FDNB and administering cyclophosphamide prior to administering DNP-conjugated tumor cells and an adjuvant, which treatment results in the claimed T-cell dependent responses. Applicant's arguments have not been found persuasive and the rejection is maintained.

6. Claims 47 and 65-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 11 , pages 12-15.

Applicant argues that, for the reasons set forth above, the rejection is in error. The argument has been considered but has not been found persuasive for the reasons set forth above. Applicant's arguments have not been found persuasive and the rejection is maintained.

7. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 12 , pages 15-18.

Applicant argues that (a) there is no reasonable expectation of successfully implementing the vaccination program described with respect to melanoma in Berd to other tumor types because this reference provides "preliminary" results that "may represent a significant advance in the immunotherapy of human melanoma and thus it lacks any reasonable expectation of an effective treatment for tumors in general or even melanoma in particular, (b)Wiseman does not supply the missing teaching and teaches an alternative form of immunotherapy that depends on the route of administration, (c) even if combined, the lack of any reasonable expectation of success from the disclosure of Berd precludes determining that the invention is obvious.

The arguments have been considered but have not been found persuasive because (a) Berd et al clearly teach that treatment of melanoma patients with autologous vaccine preceded by low dose CY induces DTH to melanoma cells and regression of metastatic tumors. The reference is specifically drawn to increasing the efficiency of the process by sensitizing with DNCB and immunizing with tumor

cells conjugated to DNP. Clearly, one would have an expectation of success since it was already known that immunization with tumor cells alone, after pretreatment with CY resulted in regression of metastatic tumors. Further, it was clearly demonstrated that a patient developed erthema, followed by ulceration and drainage of necrotic material in > 50 large dermal metastasis and that at the time of publication, some of the metastasis were beginning to regress, Further the reference specifically teaches that tumors were infiltrated with activated T lymphocytes, that the tumor masses developed a striking inflammatory response and that the patients developed DTH, (b') regardless of the route of administration, Wiseman clearly teaches that treatment of patients with lung, colon and kidney cancer with autologous tumor cell vaccine preceded by low dose CY leads to prolonged survival. It would have been expected that vaccines using other types of tumor cells, shown to effectively treat cancer, would behave in a mechanistically similar manner to the melanoma vaccine described in Berd et al where it was shown that administration of the DNP-conjugated reagent led to DHT against melanoma cells, infiltration of the tumors by activated T lymphocytes, inflammation in the tumor masses, especially in view of the fact that there is no teaching of any no distinguishing features of melanoma tumor cells which would lead one to expect that there would be a difference in the immune response to this type of tumor cell in particular, (c') Berd et al clearly demonstrate the successful use of the composition and the method. Applicant's arguments have not been found persuasive and the rejection is maintained.

8. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 13 , pages 18- 21.

Applicant argues that Berd, 1983 is cumulative to the teaching of Berd, 1989 and that Berd 1983 does not supply any of the other missing teachings that are not supplied by the combination of Berd, the antibody Patents and Geczy and that in particular, the reference does not provide any teaching concerning a haptenized tumor cell vaccine or methods of treating cancer using such a vaccine. The argument has been considered but has not been found persuasive for the reasons drawn to the lack of persuasiveness of Applicant's arguments drawn to Berd, the antibody Patents and Geczy disclosed above and further because, Berd, 1983 is cited because it is drawn to treatment of breast cancer patients with autologous vaccine. The substitution of the breast cancer cells of Berd 1983 for the melanoma cells of Berd, 1989 in the method and composition of Berd 1989 was *prima facie* obvious for the reasons set forth in Paper No. 36. Applicant has argued and discussed the references individually without clearly addressing the combined teachings. It must be remembered that the references are relied upon in combination and are not meant to be considered separately as in a vacuum. It is the combination of all of the cited and relied upon references which made up the state of the art with regard to the claimed invention. Applicant's claimed invention fails to patentably distinguish over the state of the art represented by the cited references taken in combination. In re Young, 403 F.2d 754, 159 USPQ 725 (CCPA 1968); In re Keller 642 F.2d 413,208 USPQ 871 (CCPA 1981). Applicant's arguments have not been found persuasive and the rejection is maintained.

9. Claims 43, 44, 47 and 49-76 remain rejected for the reasons previously set forth in Paper No. 36, Sections 14 , pages 21-25.

Applicant argues that (a) the deficiencies of Berd, the antibody Patents and Geczy have been addressed above, (b) Sanda and Moody fail to supply the missing teachings and propose an alternative cancer therapy and neither references provides any motivation to decorate the tumor cells with hapten in order to elicit an effective immune response. The arguments have been considered but have not been found persuasive because (a') for the reasons set forth above, (b') the combined references make the invention obvious for the reasons set forth previously and in particular Berd supplies the motivation to "decorate the tumor cells with hapten" in order to elicit an effective immune response, see above.

New Grounds of Rejection

Claim Rejections - 35 USC § 112

10. Claims 43, 49-51, 54-55 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a composition comprising the claimed tumor cell conjugate and an adjuvant, does not reasonably provide enablement for a composition comprising the claimed tumor cell conjugate alone. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The claims are drawn to treatment of malignant tumors with a composition comprising a hapten conjugated to a tumor cell. The specification teaches a melanoma vaccine administered with BCG and describes immune responses to the

melanoma vaccine administered with BCG (page 19-43) and specifically teaches in the sentence bridging pages 27-28 that "all vaccines were DNP-conjugated and mixed with BCG". It appears that the inclusion of an adjuvant may be a critical step since Livingstone et al (of record) disclosed that in a melanoma vaccine using the GM2 ganglioside, antibody responses were not induced unless BCG was added to the purified GM2 vaccine (p. 2913, paragraph bridging columns 1 and 2). Livingstone et al also state that "adjuvants ..... were important factors in the mouse studies and results of the present human trials indicate their importance in melanoma patients". Further, Hoover et al, of record, also used BCG as an adjuvant in a colorectal cancer vaccine and states that the correct amount of the appropriate adjuvant was a critical condition of the success of the immunotherapy (p. 1242, col 1, para 2). Based on the teachings above and in the specification one of skill in the art would not expect that the claimed composition could be used as contemplated for the treatment of malignant tumors without specifically including an adjuvant as demonstrated in the specification. In view of the above, one of skill in the art would be forced into undue experimentation to practice the claimed invention.

11. Claims 70 is rejected under 35 USC 112, first paragraph, as the specification does not contain a written description of the claimed invention. The limitation of cyclophosphamide administration "only" prior to the first administration of said composition has no clear support in the specification and the claims as originally filed. The specification teaches that cyclophosphamide is administered 3 days prior to each vaccine administration (p. 44, lines 26-27) but does not specifically state that the cyclophosphamide is only administered prior to vaccine. The subject matter

claimed in claims 47-58 broadens the scope of the invention as originally disclosed in the specification.

12. Claims 57, 66 and 70 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claims 57 is indefinite because there is no antecedent basis for the term "cancer" in claim 44 from which claim 57 depends.

Claim 66 is indefinite because there is no antecedent basis for the term "cancer" in claim 47 from which claim 66 depends.

Claim 70 is indefinite because there is no antecedent basis for the phrase "wherein said therapeutically effective amount of cyclophophamide" in claim 47 from which claim 70 depends. Further, the claim is indefinite as the term "cyclophophamide" appears to be a misspelling of the term cyclophosphamide.

Claim Rejections - 35 USC § 103

13. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 10 , pages 8-12 and above drawn to the rejection of claims 47 and 65-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 47 and 65-76 under 35 USC 103 are relevant to the instant

rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

14. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 11 , pages 12-15 and above drawn to the rejection of claims 47 and 65-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 47 and 65-76 under 35 USC 103 are relevant to the instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

15. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 12 , pages 15-18 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the

instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

16. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 13 , pages 18-21 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

17. Claim 77 is rejected under 35 USC 103 for the reasons previously set forth in Paper No. 36, Sections 14 , pages 21-25 and above drawn to the rejection of claims 43, 44, 47 and 49-76.

The claim is drawn to the method of claim 47 wherein said administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

The claim is obvious for the reasons previously set forth. Applicant's arguments are relevant to the instant rejection. The arguments drawn to the rejection of claims 43, 44, 47 and 49-76 under 35 USC 103 are relevant to the

instant rejection. The arguments have been considered but have not been found persuasive for the reasons set forth above.

18 All other objections and rejections recited in Paper No. 36 are withdrawn.

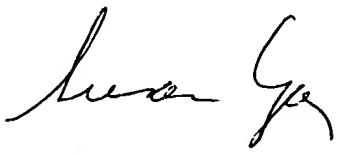
19. No claims allowed.

20. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is (703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Paula Hutzell, can be reached at (703) 308-4310. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642.



Susan Ungar
Primary Patent Examiner
March 20, 2000

Exhibit 4

08/203,004

APPLICATION NUMBER	FILING DATE	FIRST NAMED APPLICANT	ATTY. DOCKET NO.
08/203,004	02/28/94	BIRD	1225/0674

EXAMINER	
UNGAR, S	
ART UNIT	PAPER NUMBER
1642	36

HM22/0428

DARBY & DARBY PC
805 THIRD AVENUE
NEW YORK NY 10022

DUE: July 28, 1999
Docketed on 8/3 by DV for
Docketed without file ☐
Attorney

DATE MAILED: 04/28/99
10-28-99

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

OFFICE ACTION SUMMARY

☒ Responsive to communication(s) filed on 3/15/99

☐ This action is **FINAL**.

☐ Since this application is in condition for allowance except for formal matters, **prosecution as to the merits is closed** in accordance with the practice under *Ex parte Quayle*, 1935 D.C. 11; 453 O.G. 213.

A shortened statutory period for response to this action is set to expire 3 month(s), or thirty days, whichever is longer, from the mailing date of this communication. Failure to respond within the period for response will cause the application to become abandoned. (35 U.S.C. § 133). Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

Disposition of Claims

☒ Claim(s) 43-44,47-76 is/are pending in the application.
Of the above, claim(s) _____ is/are withdrawn from consideration.
☐ Claim(s) _____ is/are allowed.
☒ Claim(s) 43,44,47-49,71-76 is/are rejected.
☐ Claim(s) _____ is/are objected to.
☐ Claim(s) _____ are subject to restriction or election requirement.

Application Papers

☐ See the attached Notice of Draftsperson's Patent Drawing Review, PTO-948.
☐ The drawing(s) filed on _____ is/are objected to by the Examiner.
☐ The proposed drawing correction, filed on _____ is ☐ approved ☐ disapproved.
☐ The specification is objected to by the Examiner.
☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. § 119

☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been

☐ received.
☐ received in Application No. (Series Code/Serial Number) _____.
☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____.

☐ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e).

Attachment(s)

☒ Notice of Reference Cited, PTO-892
☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____
☐ Interview Summary, PTO-413
☐ Notice of Draftperson's Patent Drawing Review, PTO-948

Notice of References Cited

Application No.	Applicant(s)		
08/203,004	Berd		
Examiner		Group Art Unit	Page 1 of 1
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U.S. PATENT DOCUMENTS

		DOCUMENT NO.	DATE	NAME	CLASS	SUBCLASS
	A	5,290,551	3/1/94	Berd	424	88
	B	5,702,704	12/30/97	Bucala	424	137.1
	C	5,6226,843	5/6/97	Skurkovich et al	424	140.1
	D	5,008,183	4/16/91	Osther	435	5
	E	4,232,001	11/4/80	Jensen et al	424	1
	F					
	G					
	H					
	I					
	J					
	K					
	L					
	M					

FOREIGN PATENT DOCUMENTS

		DOCUMENT NO.	DATE	COUNTRY	NAME	CLASS	SUBCLASS
	N						
	O						
	P						
	Q						
	R						
	S						
	T						

NON-PATENT DOCUMENTS

		DOCUMENT (Including Author, Title, Source, and Pertinent Pages)	DATE
	U	Sanda et al (J. Cellular Biochem. Suppl., 17, Part D, p. 120	1993
	V	Wiseman et al (Western J. Med., 151:283-288)	1989
	W	Berd et al (Proc. Am. Soc. Clin. Oncol., 2:56)	1983
	X	Moody et al (J. Urol., 145:293A)	1991

A. Ungar 4/19/99

1. The Election filed March 15, 1999 (Paper No. 35) in response to the Office Action of February 1, 1999 (Paper No. 33) is acknowledged and has been entered. Claims 43, 44, 47 and 49-76 are pending in the application and Claims 24-35 have been withdrawn from further consideration by the examiner under 37 CFR 1.142(b) as being drawn to non-elected inventions. Claims 43, 44, 47 and 49-76 are currently under prosecution.

2. Applicant's election with traverse of the species of Melanoma and DNP in Paper No. 35 is acknowledged. The traversal is on the ground(s) that the no restriction of species was made in the grandparent application, Serial No. 07/520,649 filed May 8, 1990. The argument has been noted and found persuasive as drawn to the hapten species. However, the argument has not been found persuasive as drawn to the cancer type because a review of the cited application revealed that the reason that no election of species, drawn to types of cancers, was required was because no species were recited in the claims, further, the specification does not appear to be drawn to any type of cancer composition or methods of treatment, other than for melanoma. However, upon search of the elected species, it became clear that irradiated autologous cancer vaccines were well known in the art at the time the invention was made and that haptenization of tumor cell vaccines was well known, therefore there was no undue burden in the search of the species and the requirement is withdrawn. .

3. The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

4. It is noted that a priority date of February 28, 1994 has been established for the instantly claimed application serial number 08/203,004 for all claims drawn to

breast, lung, colon, kidney or prostate tumors because the earlier filed applications do not disclose compositions comprising hapten modified tumor cell compositions or methods useful for the treatment of breast, lung, colon, kidney or prostate tumors. If applicant disagrees with any rejection set forth in this office action based on examiner's establishment of the priority date set forth above, applicant is invited to submit evidence pointing to the serial number, page and line where support can be found establishing an earlier priority date.

New Grounds of Rejection

Double Patenting

5. Claims 47, 65-71, 73, 74, 76 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1 and 2 of U.S. Patent No.5,290,551 . Although the conflicting claims are not identical, they are not patentably distinct from each other because they relate to the same inventive concept. The patented claims are generic to the instant claims and render the species claims obvious as they have all the characteristics of a vaccine useful for the treatment of a malignant tumor, melanoma, in a human patient comprising irradiated autologous melanoma cell conjugated to a hapten selected from the group including DNP mixed with an immunological adjuvant wherein said immunological adjuvant is BCG and a method of treating melanoma comprising administering cyclophosphamide followed by administration of a therapeutically effective amount of the claimed vaccine. The limitations recited drawn to eliciting an inflammatory immune response against a delayed hypersensitivity response against the tumor, activated T lymphocytes that infiltrate the tumor wherein the T lymphocytes are predominantly CD8+CD4- are inherent properties of the method since the

population that is treated and the method steps recited in US Patent No. No.5,290,551 are the same as those of the instant claims. Thus, because the method of the patent comprises the same method steps as claimed in the instant invention, with the same population, the claimed method is made obvious because the method will inherently lead to eliciting an inflammatory immune response against a delayed hypersensitivity response against the tumor, activated T lymphocytes that infiltrate the tumor wherein the T lymphocytes are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). Further, the recitation of repetition of administration of the vaccine at least six times at spaced apart intervals in claim 47 does not render the claims unobvious because immunization schedules requiring administration of antigen at least six times at spaced intervals are conventional in the immunostimulation arts as demonstrated by the teaching in US Patent No. 5,702,704 (col 20, lines 12-26), No. 5,626,843 (col. 4, lines 28-38), No. 5,008,183 col 5 lines 48-54), No. 4,232,001 (col 5, lines 25-27) which all teach immunization with at least six booster injections of antigen. Although the teaching is drawn to the antibody art, it is well known that T cells must be stimulated in order to produce the detected antibodies.

Applicant's argument in Paper No. 32 drawn to claims 47 and 76 are relevant to the instant rejection. Applicant argues that claim 47 calls for treating cancer by administering human tumor cells by repeating administration at least 6 times and claim 76 claims that administration elicits predominantly CD4-CD8+ T lymphocytes and that these claims are not suggested by the claims of the '551 patent. The argument has been noted but has not been found persuasive for the reasons stated

above, that is that the 6 time repetition is conventional in the immunostimulation art and that the CD4-CD8+ limitation is an inherent property of the method.

Claim Rejections - 35 USC § 112

6. Claims 44, 47, 56-62, 65-72 and 75-76 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for a method of treating a malignant tumor in a human patient comprising administering the composition of claim 43 and BCG, does not reasonably provide enablement for treatment a malignant tumor in a human patient without administering an BCG in combination with the claimed composition. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

The claims are drawn to treatment of malignant tumors with a composition comprising a hapten conjugated to a tumor cell. The specification teaches a melanoma vaccine administered with BCG and describes immune responses to the melanoma vaccine administered with BCG (page 19-43) and specifically teaches in the sentence bridging pages 27-28 that "all vaccines were DNP-conjugated and mixed with BCG". It appears that the inclusion of the adjuvant may be a critical step since Livingstone et al (of record) disclosed that in a melanoma vaccine using the GM2 ganglioside, antibody responses were not induced unless BCG was added to the purified GM2 vaccine (p. 2913, paragraph bridging columns 1 and 2). Livingstone et al also state that "adjuvants ..... were important factors in the mouse studies and results of the present human trials indicate their importance in melanoma patients". Hoover et al, of record, also used BCG as an adjuvant in a colorectal cancer vaccine and states that the correct amount of the appropriate adjuvant was a

critical condition of the success of the immunotherapy (p. 1242, col 1, para 2). Based on the teachings above and in the specification one of skill in the art would not expect that the claimed method would be effective in treating cancer with DNP-conjugated vaccines without specifically including BCG as demonstrated in the specification. In view of the above, one of skill in he art would be forced into undue experimentation to practice the claimed invention

7. Claim 73 is rejected under 35 USC 112, fourth paragraph because it does not further limit claim 47 from which it depends.

Claim Rejections - 35 USC § 102

8. Claim 76 is rejected under 35 U.S.C. § 102(a) as being anticipated by Murphy et al, of record.

The claim is drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said administration eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-.

Murphy et al teach a method for treating melanoma comprising administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells. Although the reference does not disclose, the elicitation of T lymphocytes that infiltrate the tumor and are predominantly CD8+CD4-, the method of the prior art comprises the same method steps as claimed in the instant invention, that is, administering to the patient a composition comprising a therapeutically

effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection to the same population, thus the claimed method is anticipated because the method will inherently lead to eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993).

Applicant's arguments in Paper No. 32 drawn to the rejection of claim 76 under 35 USC 102(a) are relevant to the instant rejection. Applicant argues that new claim 76 calls for a method of treating cancer by administering hapten-conjugated human tumor cells wherein said administering elicits predominantly CD4-CD8+ T lymphocytes and the Murphy et al paper does not disclose this limitation. The argument has been noted but has not been found persuasive for the reasons disclosed above, that is that the elicitation of specific T lymphocytes that infiltrate the tumor is an inherent property of the method.

9. Claim 76 is rejected under 35 U.S.C. § 102(b) as being anticipated by Berd et al., Proc. AACR, 1989, 30:382, of record.

The claim is drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said administration eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-.

Berd et al teach a method for treating melanoma comprising administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells. Although the reference does not disclose, the elicitation of the T lymphocytes that infiltrate the tumor and are predominantly CD8+CD4-, the method of the prior art comprises the same method steps as claimed in the instant invention, that is, administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection, to the same population, thus the claimed method is anticipated because the method will inherently lead to eliciting T lymphocytes that infiltrate the tumor and that are predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993).

Applicant's arguments in Paper No. 32 drawn to the rejection of claim 76 under 35 USC 102(b) are relevant to the instant rejection. Applicant argues that new claim 76 calls for a method of treating cancer by administering hapten-conjugated human tumor cells wherein said administering elicits predominantly CD4-CD8+ T lymphocytes and the Berd et al does not disclose this limitation. The argument has been noted but has not been found persuasive for the reasons disclosed above, that is that the elicitation of specific T lymphocytes that infiltrate the tumor is an inherent property of the method.

Claim Rejections - 35 USC § 103

10. Claims 47, 65-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Murphy et al, of record draw to claim 76 in view of US Patent No. 5,702,704,

No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Berd et al., Proc. AACR, 1989, 30:382, of record and Geczy et al, of record.

The claims are drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said composition eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor and repeating said administration at least six times at spaced apart intervals, wherein said tumor cells are selected from a group including melanoma, wherein the treating is useful for treatment of cancer selected from a group including melanoma, wherein said hapten is selected from the group including DNP, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition, wherein the dose is about 300 mg/M^2, wherein the patient is sensitized with 1-fluoro-2,4-nitrobenzene prior to administration of cyclophosphamide wherein the composition comprises an adjuvant, BCG, wherein the life of the patient is prolonged, wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

Murphy et al teach as set forth above and further teach a method for treating melanoma comprising sensitizing with DNCB, administering a therapeutically effective amount of cyclophosphamide and administering a therapeutically effective

amount of autologous, irradiated DNP-conjugated melanoma cells mixed with the adjuvant BCG. Murphy et al teach as set forth above but do not teach a method wherein the vaccine is boosted at least six times at spaced intervals, the administration of 300 mg/M^2 of cyclophosphamide, prior sensitization with 1-fluoro-2,4-nitrobenzene or eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor or wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-, wherein the life of the patient is prolonged.

US Patent No. 5,702,704 (col 20, lines 12-26), No. 5,626,843 (col. 4, lines 28-38), No. 5,008,183 col 5 lines 48-54), No. 4,232,001 (col 5, lines 25-27) all teach conventional immunization schedules wherein antigen administration is repeated at least six times at spaced intervals.

Berd et al teach a successful method of treating melanoma wherein a therapeutically effective amount of cyclophosphamide, 300 mg/M^2 of cyclophosphamide, is administered prior to autologous, irradiated, DNP-conjugated melanoma cells.

Geczy et al teach that halogenated dinitrobenzenes such as 1-chloro- and 1-fluoro-2,4-dinitrobenzene are commonly used to elicit delayed hypersensitivity (p. 189, para 1).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the methods of Murphy et al and the cited patents because it is clearly conventional to repeat antigen administration at

least six times at spaced intervals. Although the teaching is drawn to the antibody art, it is well known that T lymphocytes must be stimulated in order to produce the detected antibodies. One of ordinary skill in the art at the time would have expected to successfully use the method with the conventional immunization schedule. Further, although the limitations drawn to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4- are not recited in the reference, the method of the prior art comprises the same method steps as claimed in the instant invention, to treat the same population, thus the claimed method is anticipated because the method will inherently lead to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). Further, it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to use a dose of 300 mg/M^2 of cyclophosphamide in the method of Murphy et al because Berd et al teach that the dose is therapeutically effective in a method which uses the same haptenized melanoma cells with the same population of patients. One of ordinary skill in the art would have expected to successfully use the method of Murphy with a dosage of 300 mg/M^2 of cyclophosphamide because Berd et al demonstrated the successful use of the method. Finally, it would have been *prima facie* obvious to substitute DNFB for the DNCB of Murphy et al because Geczy et

al teach that halogenated dinitrobenzenes such a 1-chloro- and 1-fluoro-2,4, dinitrobenzenes are commonly used to elicit delayed hypersensitivity and are clearly closely related haptenic molecules which function to produce the same effects and are therefore functionally equivalent. Finally, as drawn to prolonged survival, the claimed method appears to be the same as that of the prior art method absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's method with the method of the prior art in order to establish that the method of the prior art does not possess the same material structural and functional characteristics of the claimed method. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the method of the prior art does not result in prolonged survival of the patient and is functionally different than the method taught by the prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

Applicant's arguments drawn to the rejection of claims 47, 65-71, 73 and 74 in Paper No. 32 are relevant to the instant rejection.

Applicant argues that claims 47, 65-71, 73 and 74 are not anticipated by Murphy et al because claims 65-71, 73 and 74 depend from claim 47. The argument has been noted but has not been found persuasive for the reasons disclosed above.

11. Claims 47, 65-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Geczy et al, of record.

The claims are drawn to a method of treating a malignant tumor in a patient comprising administering to the patient a composition comprising a therapeutically effective amount of tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of the patient, are autologous to said patient and have been rendered incapable of growing in the body of a human upon injection therein, said composition eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor and repeating said administration at least six times at spaced apart intervals, wherein said tumor cells are selected from a group including melanoma, wherein the treating is useful for treatment of cancer selected from a group including melanoma, wherein said hapten is selected from the group including DNP, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide prior to administration of said composition, wherein the dose is about 300 mg/M^2, wherein the patient is sensitized with 1-fluoro-2,4-nitrobenzene prior to administration of cyclophosphamide wherein the composition comprises an adjuvant, BGC, wherein the administration prolongs survival of said patient wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.

Berd et al teach a method for treating melanoma comprising sensitizing with DNCB, administering 300 mg/M^2 of cyclophosphamide prior to administering a therapeutically effective amount of autologous, irradiated DNP-conjugated melanoma cells mixed with the adjuvant BCG wherein the patients are sensitized

with DNCB prior to cyclophosphamide administration. Berd et al teach as set forth but do not teach a method wherein the vaccine is boosted at least six times at spaced intervals or eliciting at least one of the following upon administration to said patient with an adjuvant: an inflammatory immune response against the tumor; a delayed-type hypersensitivity response against the tumor; and activated T lymphocytes that infiltrate the tumor or wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4- or that the administration prolongs survival of the patient.

US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 and No. 4,232,001 and Geczy et al teach as set forth above.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to combine the methods of Berd et al and the cited patents because it is clearly conventional to repeat antigen administration at least six times at spaced intervals. Although the teaching is drawn to the antibody art, it is well known that T lymphocytes must be stimulated in order to produce the detected antibodies. One of ordinary skill in the art at the time would have expected to successfully use the method with the conventional immunization schedule. Further, although the limitations drawn to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-. are not recited in the reference, the method of the prior art comprises the same method steps as claimed in the instant invention, and the same population, thus the claimed method is anticipated because the method will

inherently lead to an inflammatory immune response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor wherein the administration elicits T lymphocytes that infiltrate the tumor of said human, said lymphocytes being predominantly CD8+CD4-.. See Ex parte Novitski 26 USPQ 1389 (BPAI 1993). It would have been *prima facie* obvious to substitute DNFB for the DNCB of Berd et al because Geczy et al teach that halogenated dinitrobenzenes such a 1-chloro- and 1-fluoro-2,4, dinitrobenzenes are commonly used to elicit delayed hypersensitivity and are clearly closely related haptenic molecules which function to produce the same effects and are therefore functionally equivalent. Finally, as drawn to prolonged survival, the claimed method appears to be the same as that of the prior art method absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's method with the method of the prior art in order to establish that the method of the prior art does not possess the same material structural and functional characteristics of the claimed method. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the method of the prior art does not result in prolonged survival of the patient and is functionally different than the method taught by the prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

12. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001 and Geczy

et al, of record, as applied to claims 47, 65-76 and further in view of Wiseman et al (Western J. Med., 1989, 151:283-288).

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are selected from lung, colon and kidney, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment of lung cancer, colon cancer, or kidney cancer, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein said administration prolongs survival of the patient, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4.

Berd et al (1989) record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, and Geczy et al teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are kidney, colon, lung or wherein the cancer treated is kidney cancer, colon cancer or lung cancer.

Wiseman et al teaches compositions comprising autologous irradiated melanoma cancer cells, lung cancer cells, colon cancer cells and kidney cancer cells which are administered to treat patients with melanoma, lung cancer, colon cancer, and kidney cancer (Table 3, page 285), respectively wherein the patients were pretreated with 300 mg/M^2 cyclophosphamide (see abstract - Drug Table) wherein the patients showed increased immunological responses to the cancer (see abstract) wherein patients with, lung cancer, colon cancer, and kidney cancer all showed prolonged survival (Table 3, page 285).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the lung cancer cells, colon cancer cells or kidney cancer cells of Wiseman et al in the method of Berd et al because each of these cell types had been demonstrated to be immunogenic and to elicit responses in the respective cancer patients and because Berd et al teaches that haptenizing cancer cells increases the efficiency of the immunizing process. One of ordinary skill in the art at the time the invention was made would have been motivated to substitute the tumor cells of Wiseman et al into the method of Berd et al because Wiseman et al teach that their method prolongs survival in some cases and Berd et al teach that their method increases efficiency of the immunization process, thus increased efficiency would be expected to result in increased treatment

efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int.).

13. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al. (1989) of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied

to claims 43, 44, 47 and 49-76 and further in view of Berd et al (Proc. Am. Soc. Clin. Oncol., 1983, Vol 2:56)

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are selected from the group including breast, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment of breast cancer wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4-.

Berd et al., (1989) of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to

claims 43, 44, 47 and 49-76 teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are breast or wherein the cancer treated is breast cancer or wherein the life of the patient is prolonged.

Berd et al (1983) teach a composition and a method for the treatment of breast carcinoma comprising administration of autologous tumor cell/BCG preceded by cyclophosphamide which administration resulted in delayed-type hypersensitivity in 5 patients.

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the breast cancer cells of Berd et al (1983) in the method of Berd et al (1989) because this cell type had been demonstrated to be immunogenic and to elicit responses in the breast cancer patients and one of ordinary skill in the art would have been motivated to substitute the breast cancer cells of Berd et al (1983) in the method of Berd et al (1989) because Berd et al teaches that haptenizing cancer cells increases the efficiency of the immunizing process, thus increased efficiency would be expected to result in increased treatment efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes

that infiltrate the tumor said lymphocytes being predominantly CD8+CD4 and administration leading to prolonged survival of the patient, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int. Finally although the reference does not specifically teach irradiation of the tumor cells prior to administration, it is clear that it would have been *prima facie* obvious and one of ordinary skill in the art would have been motivated to treat the cells to prevent their growth in a body prior to administration, in order to prevent exogenously derived cancer in the patient.

14. Claims 43, 44, 47 and 49-76 are rejected under 35 U.S.C. § 103 as being unpatentable over Berd et al., Proc. AACR, 1989, 30:382, of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to claims 43, 44, 47 and 49-76 and further in view of Sanda et al (J. Cellular Biochem. Suppl. No. 17, Part D, p. 120) and Moody et al (J. Urol., 1991, 145:293A).

The claims are drawn to a composition comprising human tumor cells that are conjugated to a hapten, are of the same tumor type as a malignant tumor of a patient for whom treatment with the composition is intended, are autologous to said patient, have been rendered incapable of growing in the body of a human upon injection therein said composition eliciting an inflammatory immune response against the tumor wherein the tumor is not melanoma, a method for treating a malignant tumor in a human patient comprising administering said composition to the patient wherein said composition elicits, following administration of said composition with an adjuvant, BCG at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T lymphocytes that infiltrate the tumor, wherein the tumor cells are prostate, wherein said hapten is selected from the group including DNP and TNP, wherein the composition further comprises a carrier which is a saline solution or culture medium, wherein the method is useful for the treatment prostate cancer, wherein the method further comprises administering a therapeutically effective amount of cyclophosphamide, 300 mg/M^2, further comprising sensitizing the patient to a therapeutically effective amount of 1-fluoro-2,4, dinitrobenzene prior to administering cyclophosphamide, wherein the life of the patient is prolonged, wherein said administration elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4-.

Berd et al. (1989), of record, in view of US Patent No. 5,702,704, No. 5,626,843, No. 5,008,183 or No. 4,232,001, Geczy et al, of record, as applied to claims 43, 44, 47 and 49-76 teach as set forth above but the combined references do not teach a composition or method of treatment wherein the tumor cells are prostate

or wherein the cancer treated is prostate cancer or wherein the life of the patient is prolonged.

Moody et al (J. Urol., 1991, 145:293A) teach that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo* against rapidly growing rat prostate carcinoma induced by autologous, cells that were not transfected.

Sandra et al addressed the feasibility of gene therapy for human prostate cancer by demonstrating that retroviral vector MFG allows high efficiency transduction of human prostate cancer cells which was demonstrated in primary culture prostate cancer cells from 7 consecutive patients which demonstrates the feasibility of using MFG in genetic therapy for prostate cancer (see abstract).

It would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to substitute the prostate cancer cells of Sandra et al, transfected with lymphokine in the method of Moody et al for the melanoma cells in the method of Berd et al because Moody et al have demonstrated in an appropriate animal model that lymphokine-transfected prostate cells generate an anti-tumor effect *in vivo* against rapidly growing prostate carcinoma and because Sandra et al have demonstrated the feasibility of gene therapy for human prostate cancer by demonstrating the successful transfection of human prostate cancer cells with retroviral vector MFG. One of ordinary skill in the art would have been motivated to substitute the prostate cancer cells of Sandra et al, transfected with lymphokine in the method of Moody et al for the melanoma cells in the method of Berd et al because autologous anti-cancer cell vaccination was known to those of ordinary skill in the art and because Berd et al teach that the method that

haptenizing cancer cells increases the efficiency of the immunizing process, thus increased efficiency would be expected to result in increased treatment efficacy. Further, as drawn to claims 54-55, it is clearly obvious to include a saline carrier in a composition which is prepared for *in vivo* administration because it has been held by the Court that a compound and a carrier are obvious, if it is obvious in the art to utilize a carrier with related compounds. See *In re Rosicky*, 125 USPQ 341 (CCPA 1960). Finally, as drawn to the limitations including, eliciting an inflammatory immune response against the tumor, at least one of an inflammatory response against the tumor, a delayed-type hypersensitivity response against the tumor and activated T, elicits T lymphocytes that infiltrate the tumor said lymphocytes being predominantly CD8+CD4 and administration leading to prolonged survival of the patient, the claimed compositions and methods of treatment appear to be the same or similar to those of the combined references absent a showing of unobvious differences. The office does not have the facilities for examining and comparing applicant's product or methods with the product or methods of the combined prior art in order to establish that the product or methods of the combined prior art does not possess the same material structural and functional characteristics of the claimed product or methods. In the absence of evidence to the contrary, the burden is upon the applicant to prove that the claimed products and methods are functionally different than those taught by the combined prior art and to establish patentable differences. See In re Best, 562 F.2d 1252, 195 USPQ 430 (CCPA 1977) and Ex parte Gray, 10 USPQ 2d 1922 1923 (PTO Bd. Pat.App. & Int. Although neither the Moody et al or Sandra et al reference specifically teaches irradiation of the tumor cells prior to administration, it is clear that it would have been *prima facie* obvious and one of ordinary skill in the art would have been motivated to treat the cells to

prevent their growth in a body prior to administration, in order to prevent exogenously derived cancer in the patient.

15. If applicant disagrees with any rejection set forth in this office action based on examiner's establishment of a priority date of February 28, 1994 for the instantly claimed application serial number 08/203.004, applicant is invited to submit evidence pointing to the serial number, page and line where support can be found establishing an earlier priority date.

16. No claims allowed.

17. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Susan Ungar, PhD whose telephone number is (703) 305-2181. The examiner can normally be reached on Monday through Friday from 7:30am to 4pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Paula Hutzell, can be reached at (703) 308-4310. The fax phone number for this Art Unit is (703) 308-4242.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group receptionist whose telephone number is (703) 308-0196.

Effective, February 7, 1998, the Group and/or Art Unit location of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1642.

SUSAN UNGAR
PATENT EXAMINER

Susan Ungar

April 26, 1999

Exhibit 5

Lab Investigation 62(1): 70A 1990

407 MATURE TERATOMA IN THE LUNG FOLLOWING GONADAL TERATOCARCINOMA.

C.A. Moran and D. Carter, Yale University School of Medicine, New Haven, Ct.

Three cases in which mature teratomas were the only pulmonary manifestation of metastases from gonadal teratocarcinomas - Embryonal carcinoma & Teratoma - were found in the files of the Yale-New Haven Hospital. The patients were all men - 24, 26, and 58 years of age when orchiectomy was performed. The primary tumors each contained a prominent embryonal carcinoma component as well as elements of mature and immature teratoma. The interval from orchiectomy to appearance of pulmonary metastases was less than 4 months and chemotherapy was given prior to removal of the pulmonary metastases in all cases. Two underwent thoracotomy promptly, but the interval was 9 years in the other. In two, the lesions were solitary; the resection at 9 years contained multiple lesions. All were solid and cystic grossly and entirely mature microscopically. Dermal and mucin-secreting glandular epithelium predominated in the cystic areas; fibrous stroma was the predominant feature of the solid areas. Bronchial differentiation, which is usually a component of primary mature and immature teratomas of the lung, was not evident. The course in all cases has been indolent with none of the patients showing manifestations of disease at this time.

408 IMAGE ANALYSIS OF NUCLEOLI AND NUCLEOLAR ORGANIZING REGIONS IN PROSTATIC HYPERPLASIA, PIN, AND PROSTATIC CARCINOMA.

F. Mostofi, I. Sesterhenn, R. Becker, T. Lin, and C. Davis, Armed Forces Institute of Pathology, Washington, DC

Nucleolar orgainizing regions (NORs) are structures on short arms of chromosomes 13, 14, 15, 21, and 22. These are believed to be associated with genes involving rRNA transcription during the late interphase. NORs can be demonstrated in formalin-fixed, paraffin-embedded tissue by use of colloidal silver stains. In normal cells, only one or two of these are visible. NOR counts have been used to differentiate between benign and malignant lesions.

We studied 20 prostates exhibiting areas of hyperplasia, invasive carcinoma (PCa) and intraepithelial neoplasia (PIN). We counted 50 nuclei from each area of hyperplasia, 100 of each PCa area, and 100 from each PIN area. The measurement was carried out using Leitz-TAS system and determined the total combined area of nucleoli and NORs per nucleus. The pooled data of hyperplasia differed from those of PIN and invasive carcinoma. The pooled values of PIN differed only slightly from those of the invasive carcinomas. The determination of NORs is an objective method to differentiate PIN from hyperplasia. The impression, gained on H-E stained sections and immunopathology, that PIN is related to carcinoma is further supported by the NOR values which suggest proliferative activity similar to that of carcinomas.

409 EXPRESSION OF α ACTIN AND DESMIN IN NON-MUSCLE SARCOMAS AND SARCOMA-LIKE LESIONS.

P. Mozzicato, N. Azumi, and K. Leslie, University of Vermont College of Medicine, Burlington, VT and Georgetown University School of Medicine, Washington, DC

β actin is an ubiquitous isotype of this cytoskeletal filament and is not specific for muscle cells. Because many anti-actin antibodies detect this isotype, they are not useful in the diagnosis of sarcomas showing muscle differentiation. The development of monoclonal antibodies specific to muscle isotypes of actin (α and/or γ) and to desmin have proved useful in the diagnosis of leiomyosarcoma and rhabdomyosarcoma. We have found, however, that other mesenchymal lesions without muscle differentiation occasionally express muscle-specific actin. To further define expression of muscle-specific actin among these mesenchymal lesions, we performed immunohistochemical studies using a monoclonal antibody to smooth-muscle-specific isotype of actin (α-SM actin). In addition, monoclonal antibodies to vimentin and to desmin were used. We examined formalin-fixed, paraffin-embedded tissues from cases of malignant fibrous histiocytoma (MFH), nodular fasciitis, and fibrosarcoma. Approximately 25% of the MFH cases and virtually all of the nodular fasciitis cases showed at least focal α-SM actin and, less frequently, desmin positivity. One case of fibrosarcoma showed weak and focal α-SM actin positivity. We conclude that this "spurious" actin and desmin reactivity most likely indicates myofibroblastic differentiation in non-muscle soft tissue lesions. Furthermore, caution should be exercised when diagnosing muscle differentiation in a given mesenchymal lesion based solely on reactivity of these antibodies.

410 * DETECTION OF IMMUNOGLOBULIN (Ig) GENE UTILIZATION BY *IN SITU* HYBRIDIZATION (ISH) WITH OLIGONUCLEOTIDE PROBES

J.D. Mueller, A.A. Long*, K.J. Barrett*, R.S. Schwartz*, H.J. Wolfe, Dept. of Pathology, Laboratory of Molecular Pathology and Div. of Hematology-Oncology, *Dept. of Internal Medicine, Division of Hematology-Oncology Tufts-New England Medical Center, Boston, MA

There is very little information concerning the expressed Ig repertoire of unselected human B lymphocytes. We have developed a ISH method utilizing oligonucleotide probes (23-30 bases long) complimentary to specific mRNA sequences of the constant regions (IgM and IgG) and variable regions of human Ig heavy chain, including each of the 6 defined VH gene families and 2 individual hypervariable regions (CDRs). Probes were labelled to high specific activity ($>10^8$ cpm/ug) with S35 dATP using terminal deoxynucleotidyl transferase. Cells were prefixed in 2% paraformaldehyde and cytospun onto gelatinized slides. Human B lymphocyte clones producing Ig of known isotype and whose heavy chain nucleic acid sequences were known, were used to develop optimal ISH conditions for each individual probe. Required stringencies were achieved by varying the % formamide of hybridization and wash solutions maintaining constant temperature and salt concentrations. Clones expressing different VH families are readily distinguishable and, under high stringency, clones with sequence homology of up to 87% (2 bases difference in a 23 base sequence) may also be distinguished. In peripheral blood B lymphocytes, the frequency expression of individual VH and CDR sequences by single B cells can be enumerated with this technique, thus permitting a precise determination of the expressed repertoire of human immunoglobulin genes in normal and diseased states.

411 DETECTION OF HUMAN T-CELL LEUKEMIA VIRUS TYPE I DNA IN FORMALIN FIXED PARAFFIN EMBEDDED TISSUE BY POLYMERASE CHAIN REACTION.

K. Mukai, Y. Sato, S. Furuya, T. Kinoshita, and Y. Shimosato, National Cancer Center Research Institute, Tokyo, Japan

Definite diagnosis of adult T-cell leukemia/lymphoma (ATLL) cannot be made in cases which were diagnosed and treated before the development of serological tests for human T-cell leukemia virus type I (HTLV-I). In order to assess whether retrospective study of ATLL is feasible, detection of HTLV-I DNA in formalin fixed paraffin embedded tumor tissue of known ATLL patients was attempted using polymerase chain reaction (PCR). A crude DNA sample from a 5 micron paraffin section was used as a template for in vitro enzymatic amplification of a 120 base long sequence within the gag gene of HTLV-I. In 10 cases of ATLL with positive antibody titers for HTLV-I, the presence of HTLV-1 sequence was detected in all cases. In addition, four of five cases of clinically suspected ATLL but without serological confirmation showed positive reaction for HTLV-I.

The results indicated that PCR is a sensitive tool for detection of HTLV-I DNA in formalin fixed paraffin embedded tissue and that this method can be applied for retrospective study of ATLL.

412 TUMOR INFILTRATING T CELLS IN METASTATIC MELANOMA: INDUCTION BY IMMUNIZATION WITH AUTOLOGOUS, DNP-CONJUGATED TUMOR CELLS

G. F. Murphy, A. Radu, M. Mastrangelo, D. Berd, University of Pennsylvania & Thomas Jefferson University, Philadelphia, PA

The purpose of this study was to determine whether immune mechanisms are responsible for clinically-regressing melanoma metastases in the dermis and subcutis of patients who have been immunized with dinitrophenol (DNP)-conjugated melanoma cells. Two weeks after topical sensitization with dinitrochlorobenzene, subjects pretreated with the immunopotentiating agent cyclophosphamide were injected with vaccine consisting of $10\text{-}25X10^6$ autologous, irradiated, DNP-conjugated melanoma cells mixed with BCG. A total of 10 excisional biopsies of metastatic nodules from 7 patients were obtained coincident with evidence of clinical regression (erythema, softening, diminution in size). Whereas immune cells could not be detected in tumor nodules prior to therapy, immunohistochemical analysis of regressing lesions after immunization revealed infiltration by preponderantly T cells with cytotoxic/suppressor phenotype. All T cells expressed HLA-DR antigen, and variable numbers showed reactivity for Il-2 receptor and CD45R lymphocyte surface antigen (2H4) associated with suppressor-inducer function. Intercellular adhesion molecule-1 (ICAM-1) was expressed locally by melanoma cells, and the patterns of reactivity coincided precisely with zones of T cell infiltration. Numerous HLA-DR-positive, CD4-positive, Leu 1-negative dendritic cells infiltrated throughout the melanoma nodules. Rare clusters of natural killer cells (Leu 7, Leu 11, and NKH-1-positive) were also detected. In tumors exhibiting focal necrosis, T cells and dendritic cells were no longer prominent, and Leu M1-positive monocyte/macrophages represented the predominant inflammatory cell type.

These data indicate that tumor regression in this model involves activated T cells with potential for cytotoxic function and dendritic cells putatively capable of local antigen presentation. ICAM-1 induction appears to be associated with T cell-tumor cell adhesion induced by this novel mode of immunotherapy.

Exhibit 6

File B

US005702704A

United States Patent [19]

Bucala

[11] Patent Number: 5,702,704

[45] Date of Patent: Dec. 30, 1997

[54] **ANTIBODIES TO IN VIVO ADVANCED GLYCOSYLATION ENDPRODUCTS**

[75] Inventor: **Richard J. Bucala**, New York, N.Y.

[73] Assignee: **The Rockefeller University**, New York, N.Y.

[21] Appl. No.: **486,513**

[22] Filed: **Jun. 7, 1995**

Related U.S. Application Data

[60] Division of Ser. No. 956,849, Oct. 1, 1992, which is a continuation-in-part of Ser. No. 811,579, Dec. 20, 1991, abandoned.

[51] **Int. Cl.**[6] **C07K 16/18**; A61K 39/395

[52] **U.S. Cl.** **424/137.1**; 424/152.1; 424/172.1; 530/387.5; 530/388.2; 530/388.25; 530/389.1; 530/389.3; 530/391.3

[58] **Field of Search** 530/387.5, 388.2, 530/389.25, 389.1, 389.3, 391.3; 424/137.1, 152.1, 172.1

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(List continued on next page.)

Primary Examiner—Christina Y. Chan
Assistant Examiner—Emma Cech
Attorney, Agent, or Firm—Klauber & Jackson

[57] **ABSTRACT**

The circulating advanced glycosylation endproducts Hb-AGE, serum AGE-peptides and urinary AGE-peptides are disclosed as long term markers of diseases and dysfunctions having as a characteristic the presence of a measurable difference in AGE concentration. Diagnostic and therapeutic protocols taking advantage of the characteristics of these AGEs are disclosed. Antibodies which recognize and bind to in vivo-derived advanced glycosylation endproducts are also disclosed. Methods of using these antibodies as well as pharmaceutical compositions are also disclosed, along with numerous diagnostic applications, including methods for the measurement of the presence and amount of advanced glycosylation endproducts in both plants and animals, including humans, as well as in cultivated and systhesized protein material for therapeutic use.

15 Claims, 12 Drawing Sheets

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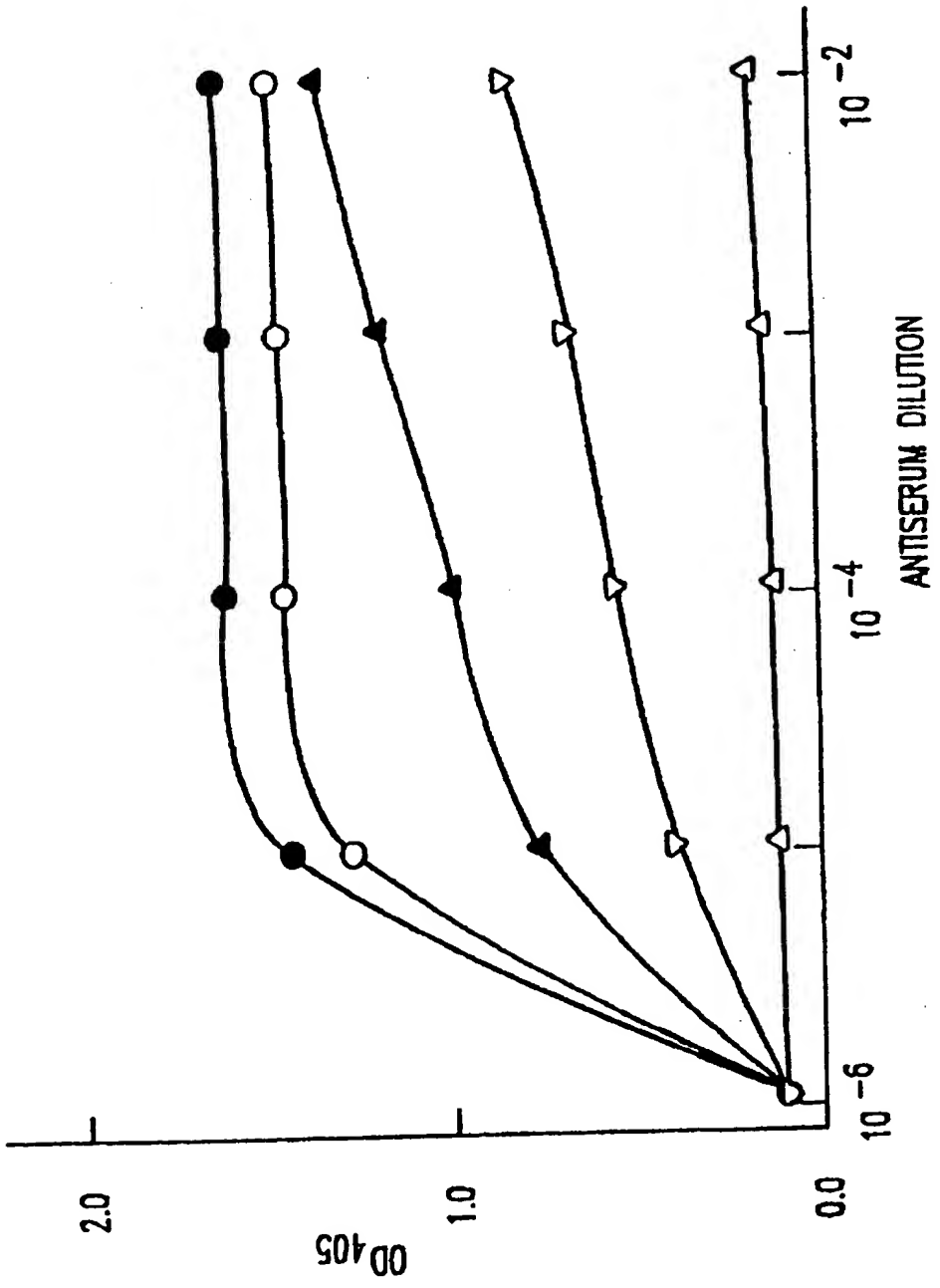

FIG. 1

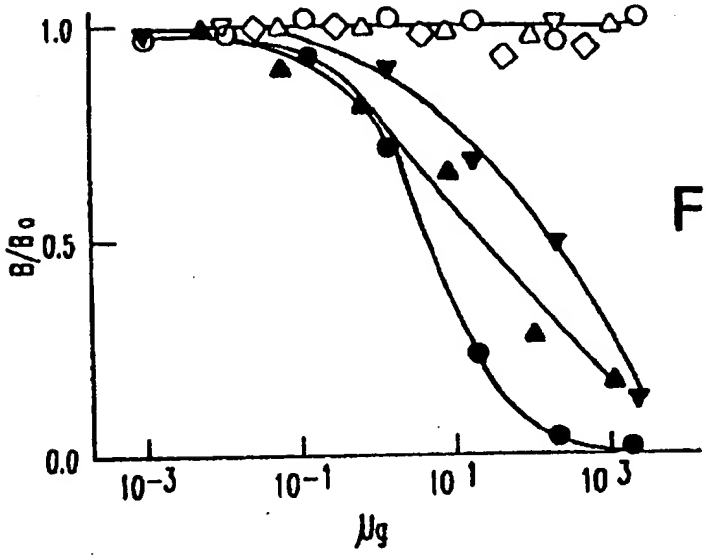

FIG.2A

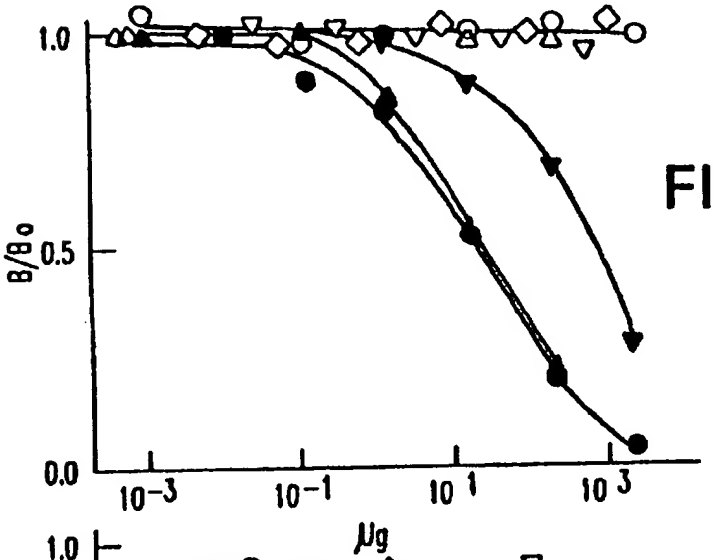

FIG.2B

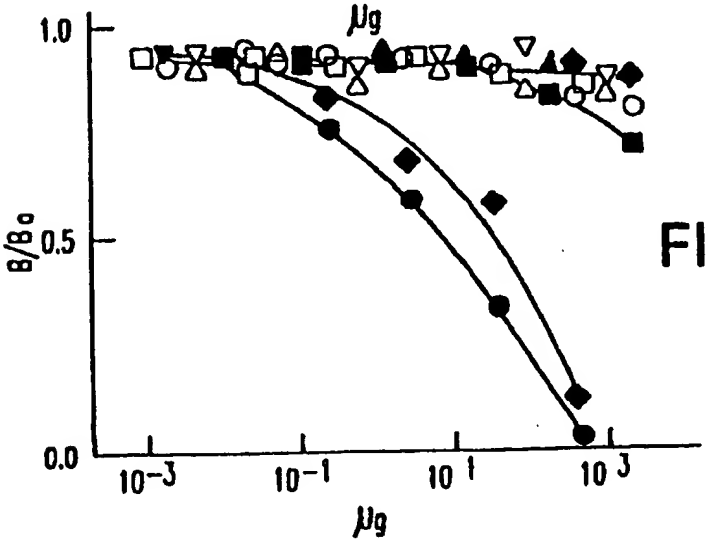

FIG.2C

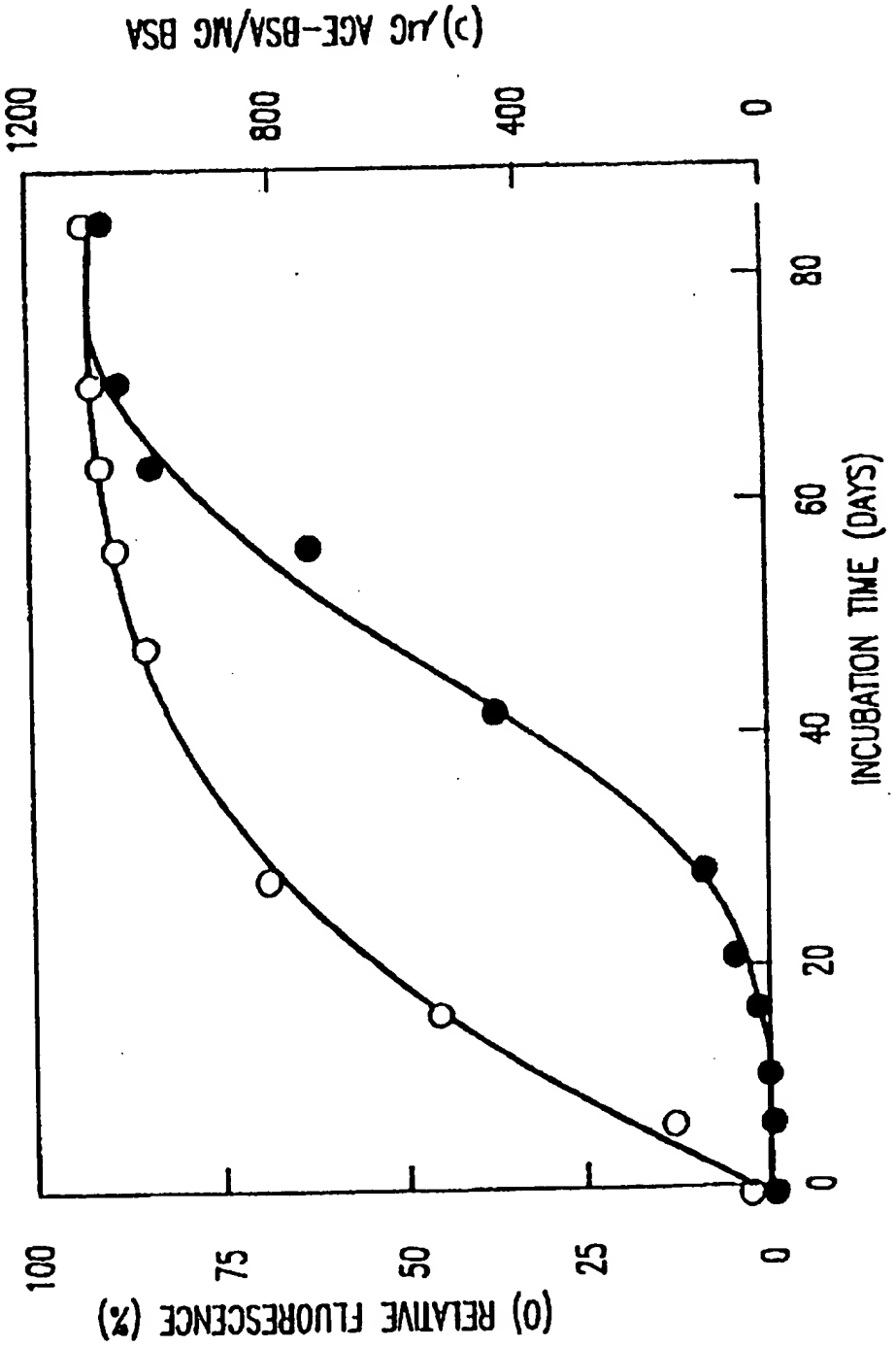
(○) μG ACE-BSA/MG BSA
1200
800
400
0
(●) RELATIVE FLUORESCENCE (%)
100
75
50
25
0
0
20
40
60
80
INCUBATION TIME (DAYS)

FIG.3

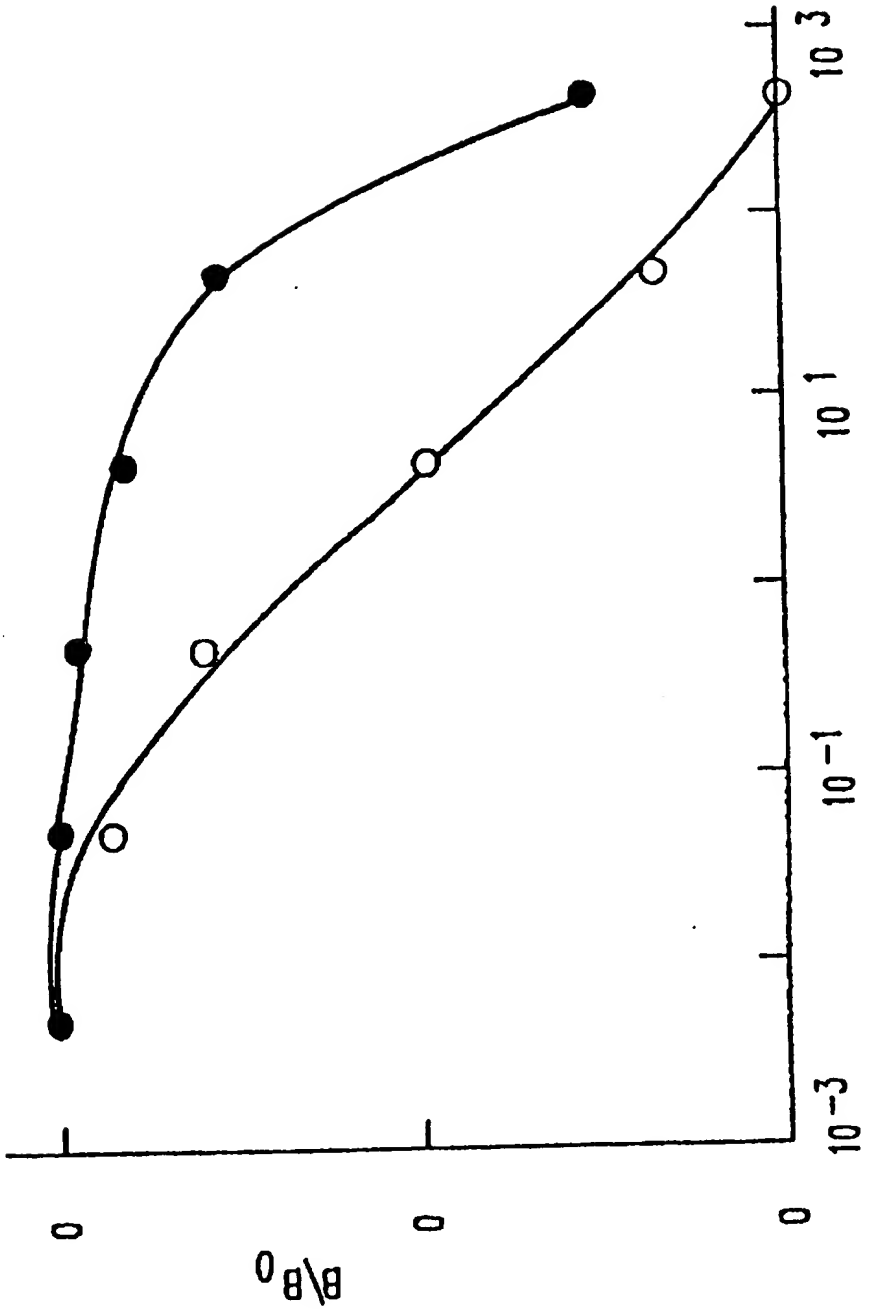

FIG.4

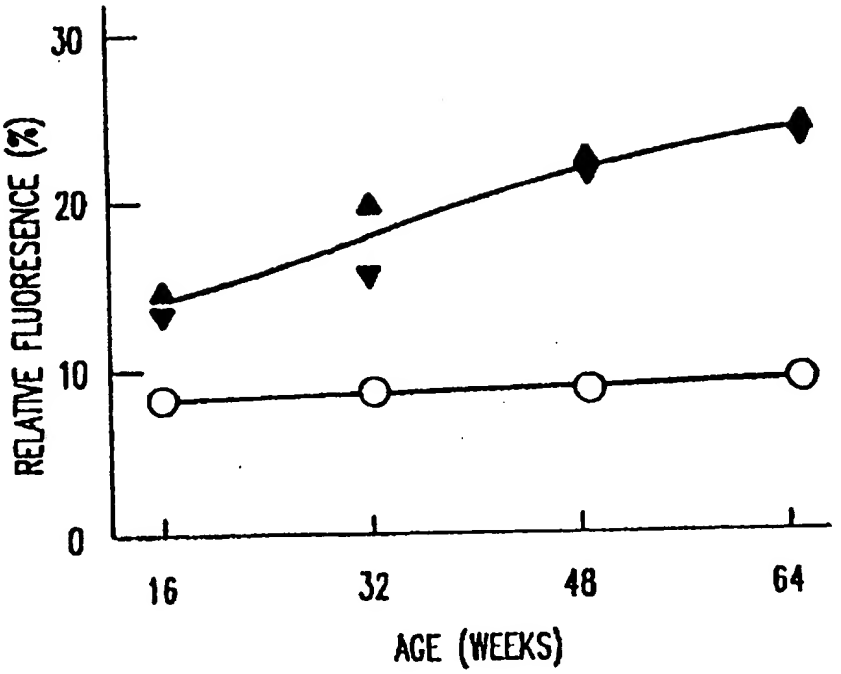
30
20
10
0
RELATIVE FLUORESCENCE (%)
16
32
48
64
AGE (WEEKS)

FIG.5A

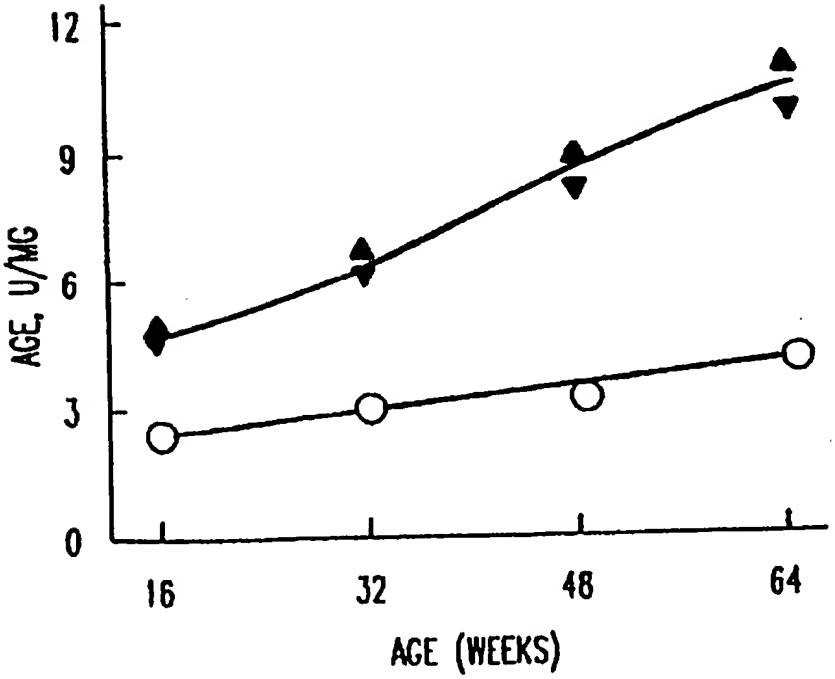
12
9
6
3
0
AGE, U/MG
16
32
48
64
AGE (WEEKS)

FIG.5B

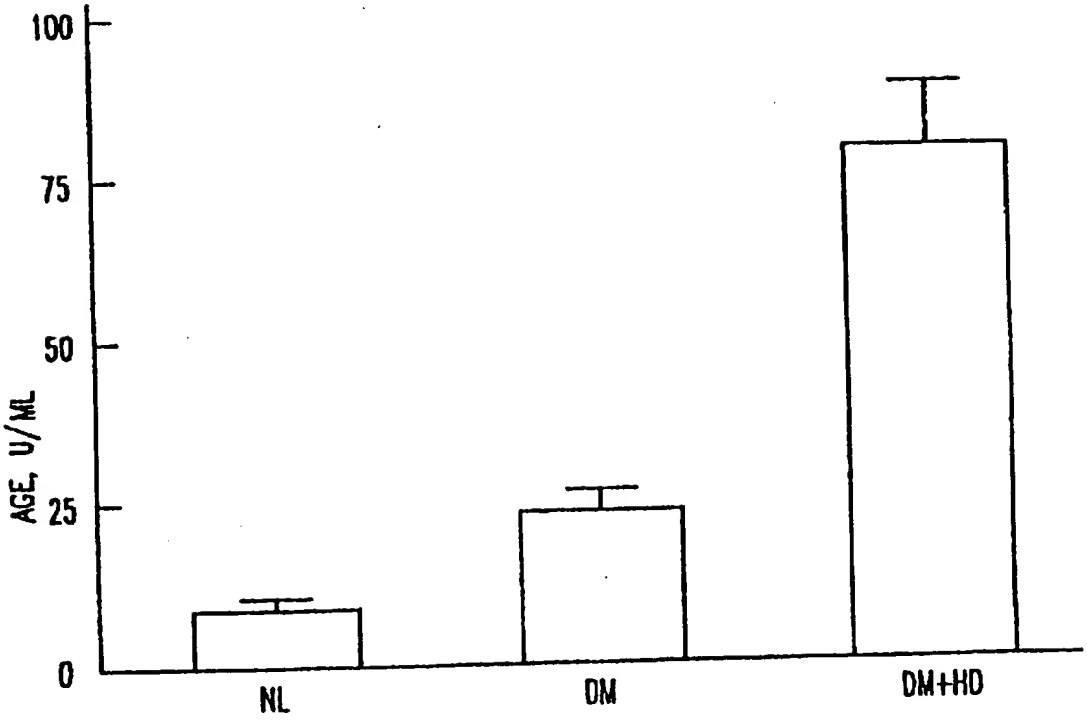

FIG.6

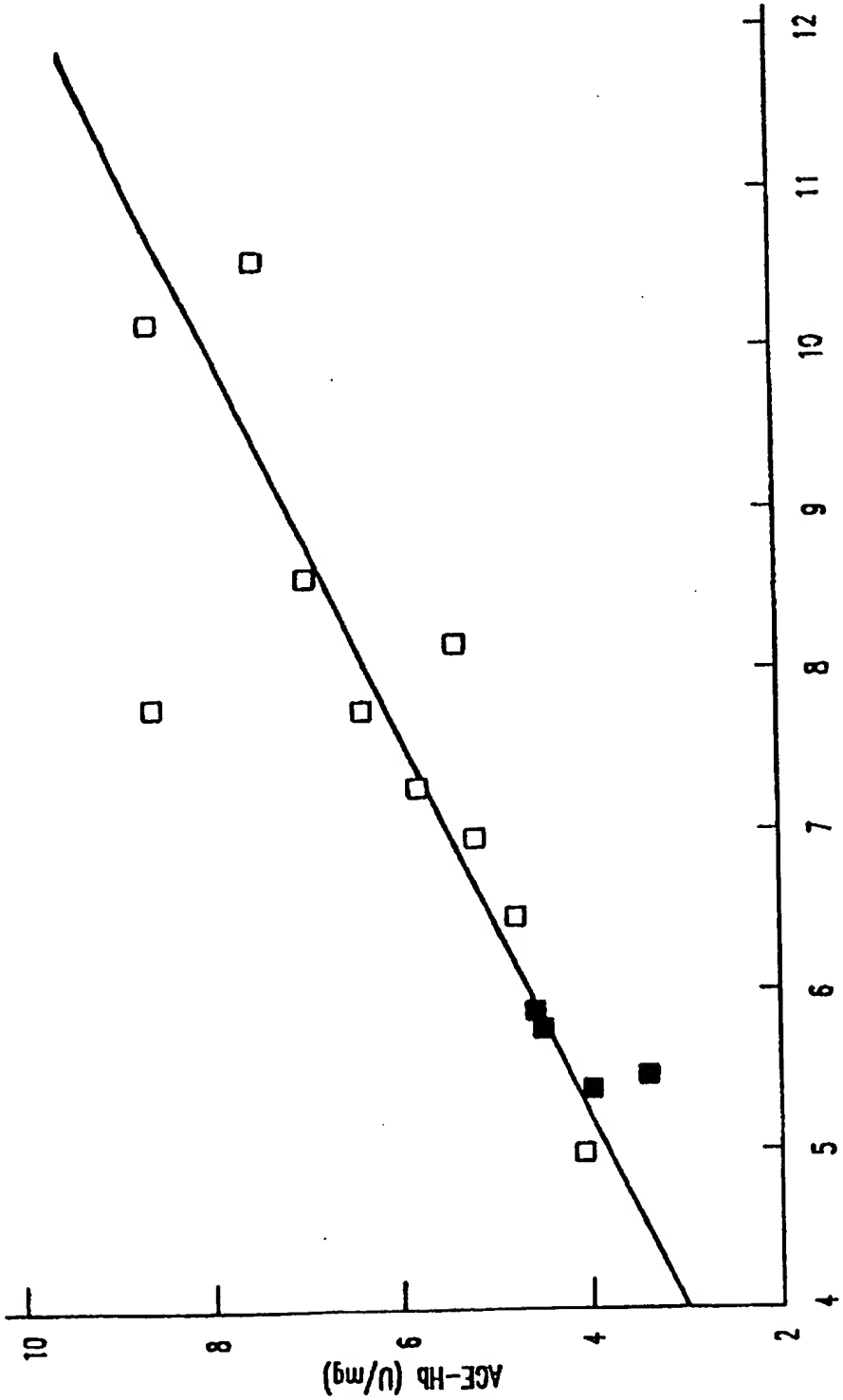

FIG.7

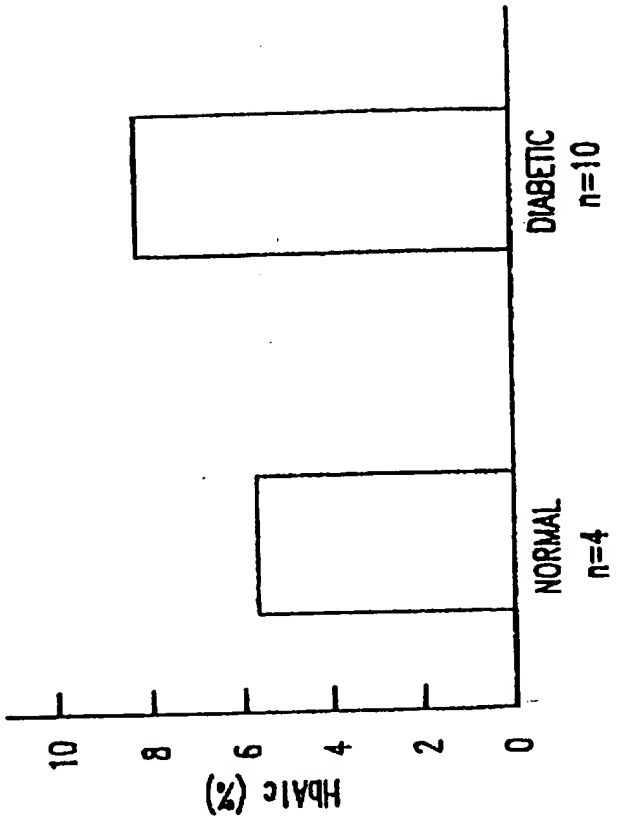
DIABETIC
n=10
NORMAL
n=4
0
2
4
6
8
10
HbA1c (%)

FIG.8B

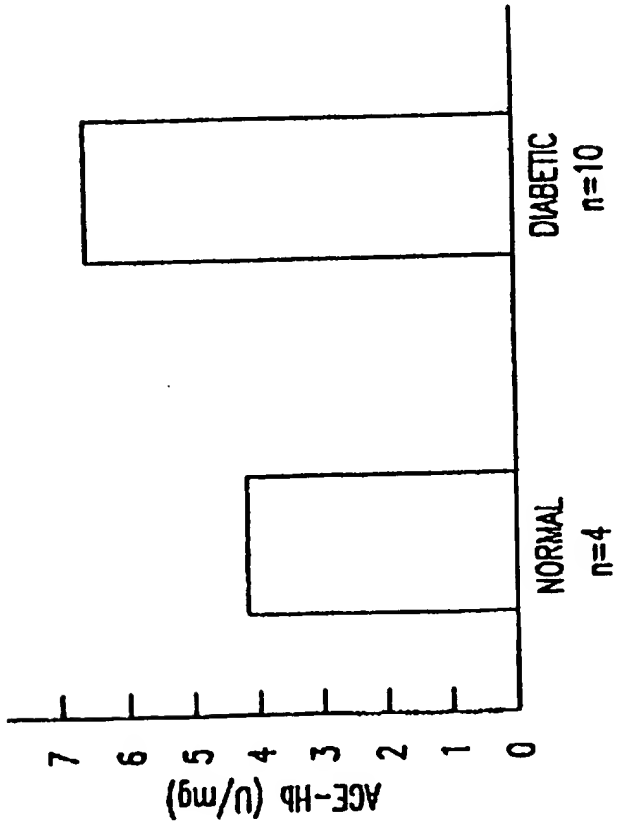
DIABETIC
n=10
NORMAL
n=4
0
1
2
3
4
5
6
7
AGE-Hb (U/mg)

FIG.8A

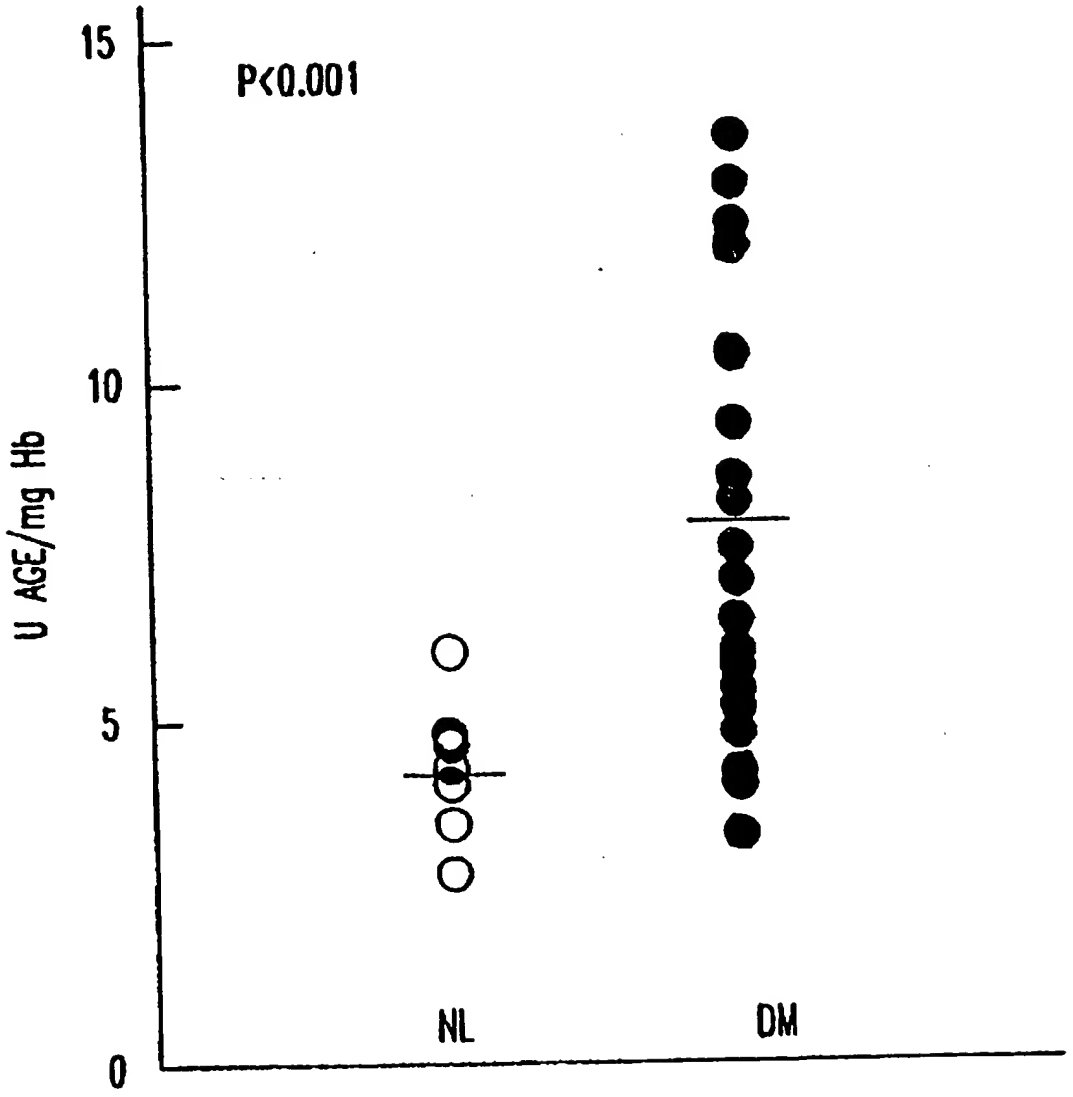

FIG.9

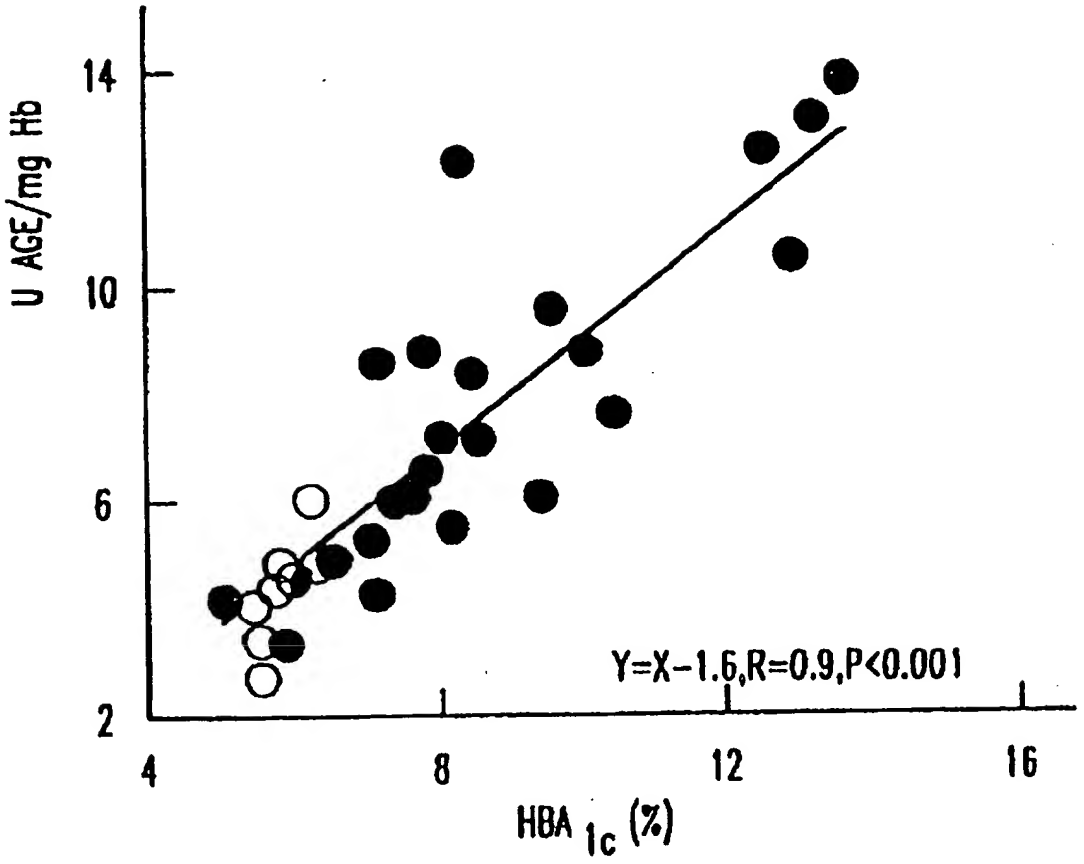

FIG. 10

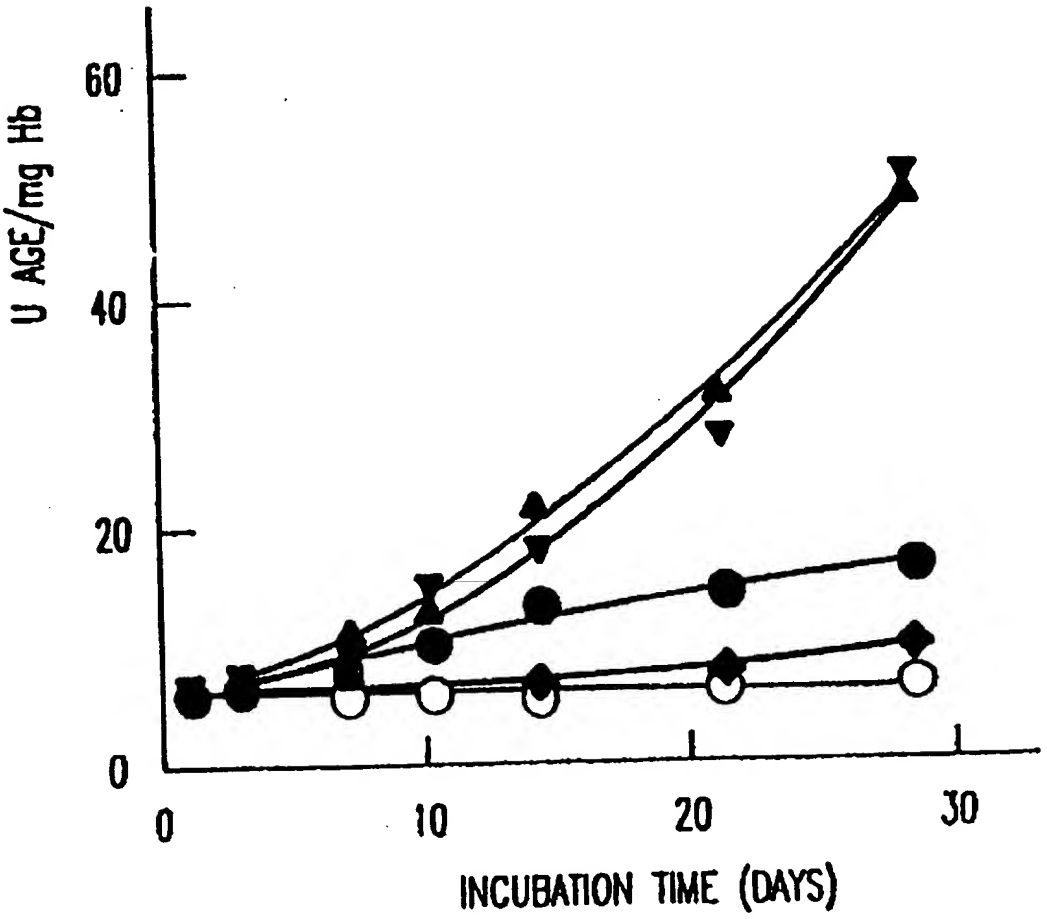

FIG. 11

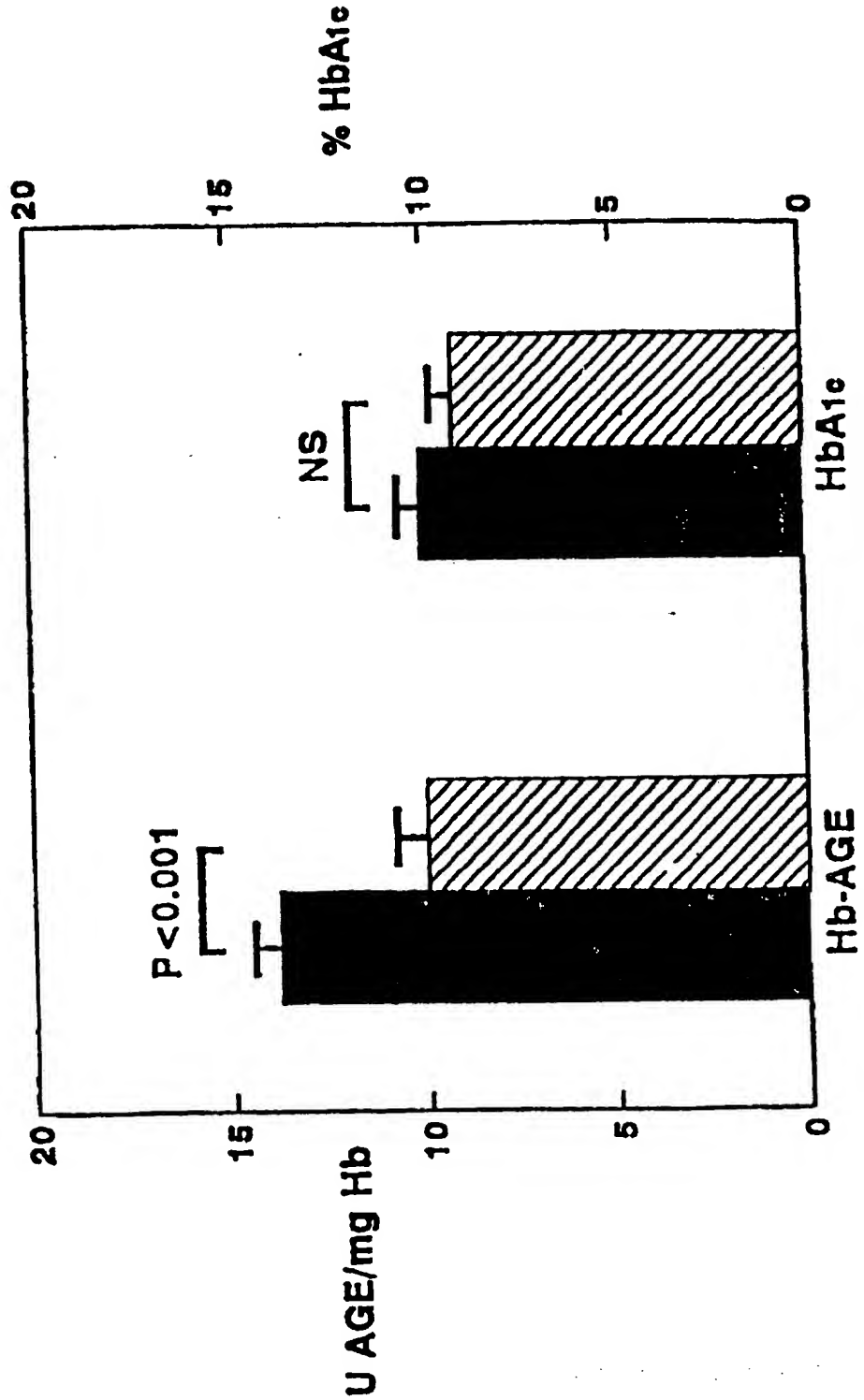

FIG. 12

ANTIBODIES TO IN VIVO ADVANCED GLYCOSYLATION ENDPRODUCTS

CROSS-REFERENCE TO RELATED APPLICATION

The present Application is a Division of application Ser. No. 07/956,849 filed Oct. 1, 1992, pending, which is a Continuation-In-Part of application Ser. No. 07/811,579, filed Dec. 20, 1991, now abandoned, by the inventor herein. Priority under 35 U.S.C. §120 is claimed as to the above earlier filed Application, and the disclosure thereof is incorporated herein by reference.

RELATED PUBLICATIONS

The following articles are noted as they are generally directed to the subject matter of the present invention: "FUNCTION OF MACROPHAGE RECEPTOR FOR NONENZYMATICALLY GLYCOSYLATED PROTEINS IS MODULATED BY INSULIN LEVELS", Vlassara, Brownlee and Cerami, DIABETES (1986), Vol. 35 Supp. 1, Page 13a; "ACCUMULATION OF DIABETIC RAT PERIPHERAL NERVE MYELIN BY MACROPHAGES INCREASES WITH THE PRESENCE OF ADVANCED GLYCOSYLATION ENDPRODUCTS", Vlassara, H., Brownlee, M., and Cerami, A. J. EXP. MED. (1984), Vol. 160, pp. 197–207; "RECOGNITION AND UPTAKE OF HUMAN DIABETIC PERIPHERAL NERVE MYELIN BY MACROPHAGES", Vlassara, H., Brownlee, M., and Cerami, A. DIABETES (1985), Vol. 34, No. 6, pp. 553–557; "HIGH-AFFINITY-RECEPTOR-MEDIATED UPTAKE AND DEGRADATION OF GLUCOSE-MODIFIED PROTEINS: A POTENTIAL MECHANISM FOR THE REMOVAL OF SENESCENT MACROMOLECULES", Vlassara H., Brownlee, M., and Cerami, A., PROC. NATL. ACAD. SCI. U.S.A. (September, 1985), Vol. 82, pp. 5588–5592; "NOVEL MACROPHAGE RECEPTOR FOR GLUCOSE-MODIFIED PROTEINS IS DISTINCT FROM PREVIOUSLY DESCRIBED SCAVENGER RECEPTORS", Vlassara, H., Brownlee, M., and Cerami, A. JOUR. EXP. MED. (1986), Vol. 164, pp. 1301–1309; "ROLE OF NONENZYMATIC GLYCOSYLATION IN ATHEROGENESIS", Cerami, A., Vlassara, H., and Brownlee, M., JOURNAL OF CELLULAR BIOCHEMISTRY (1986), Vol. 30, pp. 111–120; "CHARACTERIZATION OF A SOLUBILIZED CELL SURFACE BINDING PROTEIN ON MACROPHAGES SPECIFIC FOR PROTEINS MODIFIED NONENZYMATICALLY BY ADVANCED GLYCOSYLATION END PRODUCTS", Radoff, S., Vlassara, H. and Cerami, A., ARCH. BIOCHEM. BIOPHYS. (1988), Vol. 263, No. 2, pp. 418–423; "ISOLATION OF A SURFACE BINDING PROTEIN SPECIFIC FOR ADVANCED GLYCOSYLATION ENDPRODUCTS FROM THE MURINE MACROPHAGE-DERIVED CELL LINE RAW 264.7", Radoff, S., Vlassara, H., and Cerami, A., DIABETES, (1990), Vol. 39, pp. 1510–1518; "TWO NOVEL RAT LIVER MEMBRANE PROTEINS THAT BIND ADVANCED GLYCOSYLATION ENDPRODUCTS: RELATIONSHIP TO MACROPHAGE RECEPTOR FOR GLUCOSE-MODIFIED PROTEINS", Yang, Z., Makita, Z., Horii, Y., Brunelle, S., Cerami, A., Sehajpal, P., Suthanthiran, M. and Vlassara, H., J. EXP. MED., Vol. 174, pp. 515–524; "HUMAN AND RAT MESANGIAL CELL RECEPTORS FOR GLUCOSE-MODIFIED PROTEINS: POTENTIAL ROLE IN KIDNEY TISSUE REMODELLING AND DIABETIC NEPHROPATHY", Skolnik, E., Yang, Z., Makita, Z., Radoff, S., Kirstein, M., and Vlassara, H., J. EXP. MED., Vol. 174, pp. 931–939; and "HEMOGLOBIN-AGE: A CIRCULATING MARKER OF ADVANCED GLYCOSYLATION", Makita, Z., Vlassara, H., Rayfield, E., Cartwright, K., Friedman, E., Rodby, R., Cerami, A., and Bucala, R., SCIENCE, (In Press). All of the foregoing publications and all other references cited herein are incorporated by reference.

BACKGROUND OF THE INVENTION

The present invention relates generally to the detection and measurement of nonenzymatically glycosylated proteins, and particularly to methods and associated materials for the detection and measurement of proteins that have been nonenzymatically glycosylated in vivo.

Reducing sugars, e.g., glucose, have been shown to react non-enzymatically with protein amino groups to form a diverse series of protein bound moieties with fluorescent and crosslinking properties. These compounds, called advanced glycosylation endproducts ("AGEs"), have been implicated in the structural and functional alteration of proteins during aging and in certain diseases, e.g., long-term diabetes. Several AGEs have been identified on the basis of de novo synthesis and tissue isolation procedures.

The reaction between reducing sugars and the free amino groups of proteins initiates the post-translational modification process called advanced glycosylation. This process begins with a reversible reaction between the reducing sugar and the amino group to form a Schiff base, which proceeds to form a covalently-bonded Amadori rearrangement product. Once formed, the Amadori product undergoes further rearrangement to produce AGEs.

Because these reactions occur slowly, proteins may accumulate significant amounts of Amadori products before accumulating a measurable amount of AGEs in vivo. These AGEs can cause protein crosslinking, which in turn may reduce the structural and/or functional integrity of organs and organ parts, thus ultimately reducing or impairing organ function.

The advanced glycosylation process is particularly noteworthy in that it occurs in proteins with long half-lives, such as collagen and under conditions of relatively high sugar concentration, such as in diabetes mellitus. Numerous studies have suggested that AGEs play an important role in the structural and functional alteration which occurs in proteins during aging and in chronic disease.

Additionally, advanced glycosylation endproducts are noted to form more rapidly in diabetic, galactosemic and other diseased tissue than in normal tissue. Certain advanced glycosylation endproducts are believed to have in common a characteristic yellow-brown pigmentation, a characteristic fluorescence spectrum and the ability to form protein-protein crosslinks. AGEs form in vivo and have been isolated from naturally glycosylated material. These products are present in low abundance, are structurally heterogeneous and are labile to chemical reduction and hydrolysis. De novo synthesis and isolation procedures have led to the identification of several AGEs, such as 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole ("FFI"); 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde ("Pyrraline"); 1-alkyl-2-formyl-3,4-diglycosyl pyrrole ("AFGP"), a non-fluorescent model AGE; carboxymethyllysine; and pentosidine. However, the in vivo formation of AGEs is not limited to these precise chemical compounds, and newly discovered AGEs are addressed herein.

The study of specific AGEs synthesized in vitro in the past has necessitated the use of chemical reduction and hydroly-

sis procedures. This has left open the possibility that naturally occurring AGEs would include other compounds with alternative structures which differ from the model compounds which have been isolated.

Efforts have also been made to develop antibodies to in vivo AGEs, however no instances of success are known or have been reported. Thus, Nakayama et al., *BIOCHEM. BIOPHYS. RES. COMM.*, 162:2, pp. 740–745 (1989) studied protein bound AGEs and in particular, raised antisera against AGE-KLH derived from in vitro glycosylation. These antisera exhibited high affinity binding, and the serial dilution curves of in vitro-formed AGE-BSA, AGE-HSA and AGE-RNAse A were noted to parallel each other, suggesting that a structure in common among these particular AGE-proteins is recognized by the antisera. Further study to determine whether the structure recognized stems from advanced Maillard reactions or from the early-stage compounds, such as Schiff base adducts and Amadori rearrangement products were conducted using a number of reducing agents. Treatment with a reducing agent did not decrease immunoreactivity, and FFI was not recognized by the antibodies. Importantly, the antibodies prepared and tested by Nakayama et al. were not determined to react with AGEs formed in vivo.

Horiuchi et al., *J. BIOL. CHEM.*, 266(12), pp. 7329–7332 (1991) prepared polyclonal and monoclonal antibodies against in vitro-derived AGE-bovine serum albumin. The Horiuchi et al. antibodies also recognized in vitro-derived AGE-human serum albumin and AGE-hemoglobin, but did not recognize unmodified counterparts. Treatment of these AGE proteins with a reducing agent had no effect on immunoreactivity. Like the antibodies of Nakayama et al., the antibodies prepared by Horiuchi et al. were not determined to react with in vivo-formed AGEs.

Accordingly, despite the facility with which antibodies have been prepared in the art, the reactivity of such antibodies with in vivo-formed AGEs has not been previously achieved. The preparation of such antibodies is desirable as it makes possible the development and implementation of diagnostic and therapeutic protocols addressing the formation of advanced glycosylation endproducts in mammals including humans.

In this context, parent application Ser. No. 07/811,579, abandoned, discloses the preparation of an antiserum that contains antibodies reactive with in vivo-formed advanced glycosylation endproducts. Among the advanced glycosylation endproducts against which antibodies were raised, the reaction product of hemoglobin and a reducing sugar (Hb-AGE) was included. In addition, data were presented that compared this AGE favorably with HbA_{1c} in terms of its use as a diagnostic agent.

The present application seeks to present further data cumulative on the activity of Hb-AGE, thereby emphasizing its expanded capabilities. Also, the role of serum- and urinary AGE peptides as markers of disease and dysfunction is further elaborated herein.

SUMMARY OF THE INVENTION

In a first aspect of the present invention, the advanced glycosylation endproduct involving a complex with hemoglobin, referred to hereinafter as Hb-AGE, is reviewed in further detail and particularly with regard to its capability as a diagnostic tool. Accordingly, Hb-AGE exhibits a broad range of activity, in that Hb-AGE and/or its antibodies may be used not only in detecting the onset of either glycemic conditions or diabetes mellitus, but also in the long term monitoring of the course of such conditions, to detect fluctuations in the intensity of such conditions. Likewise, therapeutic applications for both Hb-AGE and its antagonists are contemplated.

The present invention also extends to the measurement of urinary- and serum AGE-peptides and to the diagnostic and therapeutic applications that follow. Urinary AGE-peptide levels are indicative of the turnover of tissue AGEs and are therefore also useful in the evaluation of diabetes and diabetic complications, and particularly, the control of blood glucose levels, both short term and long term, e.g., up to about 60–90 days. Serum AGE-peptides are predictive of renal disease, and particularly may be measured to determine glomerular filtration rate (GFR). Accordingly, diabetic complications involving the kidney can be monitored and serum AGE-peptides can be used to evaluate the effect of test compounds on the kidney and the level of AGEs elsewhere in the body. Serum peptide-AGEs are also useful for detecting the AGE-inducing or -forming effect of drugs, and in evaluating the therapeutic effect of AGE inhibitors, since serum peptides are derived from long-lived proteins.

Accordingly, the present invention relates to the measurement of Hb-AGE, serum AGE-peptides and urinary AGE-peptides, and to the associated methods for both the long-term and short-term monitoring of conditions involving either sugar concentrations or AGE concentrations. The invention also includes the measurement of mammalian and particularly human serum albumin AGEs (AGE-HSA), as well as food products for the assessment of spoilage, and proteins including recombinant preparations, that are intended for use as therapeutic agents. In this last mentioned connection, the invention includes the use of the present AGE antibodies in a method for monitoring the purity of such protein preparations, and a related method for the purification of the preparations that remove glycosylated proteins therefrom. Such a method would limit the unwanted administration of glycosylated proteins that are known to be clinically active in a manner deleterious to the host.

The method of assessing the presence or activity of disease in which Hb-AGE is the marker comprises obtaining a blood or other serum sample from a mammal, determining the presence or amount of Hb-AGE in the sample and comparing this amount to a standard.

Hb-AGE measurements provide an appropriate index of long-term tissue modification by AGEs and are useful in assessing the contribution of advanced glycosylation to a variety of diabetic and age-related complications. While hemoglobin A_{1c} (HbA_{1c}) has been reported as predictive of the extent of glycation on the hemoglobin β chain, HbA_{1c} is only an intermediate in the advanced glycosylation pathway and numerous other intermediates are believed to exist. Moreover, HbA_{1c} is not predictive of pathology. Therefore, Hb-AGEs levels are believed to be a better measure of disease, drug effectiveness, etc. Hb-AGEs are used in the present invention to more readily correlate to the progression of disease and longer term control of blood sugar levels, which is greater than about 3–4 weeks. The reduction in Hb-AGE levels as a result of aminoguanidine therapy is a primary example of the detection of successful pharmacological inhibition of advanced glycosylation in human subjects.

The invention also extends to the embodiment thereof that is common to the present disclosure and that of parent application Ser. No. 07/811,579, abandoned, concerning antibodies which react with in vivo-produced advanced glycosylation endproducts. Included therefore, is an antiso-

rum that contains antibodies reactive with in vivo-formed advanced glycosylation endproducts and has the following characteristics:

A. it reacts with an immunological epitope common to in vivo-formed advanced glycosylation endproducts;

B. it is cross reactive with advanced glycosylation endproducts formed in vitro; and

C. it is not cross reactive with the following advanced glycosylation endproducts however formed: FFI, AFGP, pyrraline, carboxymethyllysine, and pentosidine. Particularly, the common epitope is formed by the incubation of a reducing sugar with a proteinaceous material selected from the group consisting of RNAse, lysine, hemoglobin, collagen Type IV, LDL, BSA and HSA.

The antibodies of the invention may be polyclonal or monoclonal, and if the latter, may be prepared by the hybridoma method, or other known recombinant techniques. As illustrated herein, the antibodies may be raised in an immunocompetent mammal by hyperimmunizing said mammal with AGEs or a protein on which AGEs have been formed. The antibodies produced recognize and bind to in vivo-formed AGEs, samples which contain such AGEs, e.g., diabetic tissue or serum, as well as in vitro-formed AGEs which form on proteins as a result of incubation with sugars.

The anti-AGE antibodies of the invention are likewise characterized in that they do not recognize certain AGEs that have been synthetically produced, e.g., FFI, pyraline, AFGP, carboxymethyllysine and pentosidine.

The anti-AGE antibodies described herein are further characterized as follows:

(a) the antibodies can be formed by hyperimmunization of a mammal with AGE-RNAse;

(b) the antibodies are reactive with the following AGEs: AGE-RNAse, AGE-hemoglobin, AGE-BSA, AGE-HSA, AGE-collagen IV, AGE-LDL and AGE-lysine;

(c) the antibodies are non-reactive with unmodified HSA or BSA, FFI-BSA, formylated-albumin, malcylated-albumin, LDL, collagen IV, acetyl-LDL, FFI, AFGP, pyrraline, carboxymethyllysine, pentosidine, lysine, deoxypropylaminofructose or deoxymorpholinofructose.

In a further aspect of the invention, the present anti-AGE antibodies may be recovered from an antiserum raised in a suitable host which has been inoculated with a particular AGE-protein. The preferred method comprises administering to an immunocompetent mammal an effective amount of an AGE or a compound containing AGEs as above described, to induce the formation of the present anti-AGE antibodies, and obtaining from the mammal a serum which contains the anti-AGE antibodies. A particular AGE-protein comprises the product of the incubation of RNAse with glucose.

Another aspect of the present invention relates to immunological assays for detecting the presence or quantity of AGEs in a sample, comprised of:

(a) binding a sample suspected of containing AGEs, an AGE carrier, anti-AGE antibodies or another AGE binding partner to a solid support;

(b) contacting the specie attached to the solid support with an analyte to be tested for the presence or quantity of AGEs, anti-AGE antibodies or other AGE binding partner;

(c) labelling the AGEs, anti-AGE antibodies or other binding partner with a detectible label; and

(d) comparing the amount of bound label to a standard.

The above assay format may be adapted to examine samples of plant and animal matter for the presence of AGEs, for example to detect the likelihood or onset of food spoilage, and the present invention is intended to extend to this utility.

The invention further encompasses the use of the present antibodies for the detection of disease in a mammal, which is characterized in that an abnormal level of AGEs such as Hb-AGE, serum AGE peptides and urinary AGE peptides, is present. The antibodies may be either polyclonal or monoclonal, and are as characterized earlier herein.

Assay kits are also encompassed which are useful for performing the assay/diagnosis described herein, which include suitable reagents for detecting or quantifying AGEs such as Hb-AGE, urinary- and serum AGE-peptides, AGE-antibodies or other AGE binding partners in a sample.

Therapeutic compositions and methods of use in the prevention, diagnosis or treatment of disease using these compositions are also included, wherein an effective amount of the composition is administered to a patient in need of such treatment.

Consequently, a primary object of the present invention is to provide an antiserum which contains antibodies which recognize and bind to in vivo-formed AGEs, and a method of making the antiserum containing anti-AGE antibodies which have not heretofore been produced.

Another object of the invention described herein is to provide immunochemical assay protocols using the antiserum described above, for proteins which are modified by advanced glycosylation. AGE-immunogens have been prepared in vitro without the use of chemical reduction and hydrolysis procedures, and antisera have been produced in vivo using these AGEs.

Another object of the present invention is to provide immunoassay protocols which encompass the use of polyclonal antibodies raised in response to an immunogenic challenge with AGEs, as well as monoclonal antibodies all of which are specific to in vivo AGE epitopes.

It is a further object of the present invention to provide a method for measuring advanced glycosylation endproducts in a variety of biological samples that is rapid and reliable, taking advantage of the anti-AGE antibodies which have been raised and characterized.

It is a further object of the present invention to provide kits containing suitable reagents for the measurement of AGEs including as the internal standard a material selected from Hb-AGE, serum AGE-peptides and urinary AGE-peptides, which are suited to a broad range of alternative immunological protocols.

It is a still further object of the present invention to provide a method for the long term monitoring of glycemic conditions including diabetes, by the measurement of AGEs such as Hb-AGE, serum AGE-peptides and urinary AGE-peptides, that is fast and reliable.

These and other objects will be apparent to the ordinarily skilled artisan from a review of the detailed description taken in conjunction with the following drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is an antiserum dilution curve for anti-AGE-RNAse antiserum. Antiserum was titered in a non-competitive ELISA utilizing the following absorbed antigens: RNAse (o), Glucose-derived AGE-RNAse (●), BSA (Δ), Glucose-derived AGE-BSA (▲), and G6P-derived AGE-BSA (▼);

FIG. 2 is an ELISA-competition curve for anti-AGE-RNAse antiserum. Assays were performed as described in

Materials and Methods and employed glucose-derived AGE-BSA as the absorbed antigen. All points represent the mean of triplicate determinations.

(A): Reaction with a variety of modified albumins. Glucose-derived AGE-BSA (●), G6P-derived AGE-BSA (▲), Fructose-derived AGE-BSA (▼), FFI-BSA (Δ), Formylated-BSA (▽), Maleyl-BSA (◇), and BSA (○).

(B): Reaction with AGE-modified and unmodified proteins. G6P-derived AGE-HSA (●), Glucose-derived AGE-LDL (▲), Glucose-derived AGE-collagen IV (♦), Glucose-derived AGE-RNAse (■), HSA (○), LDL (Δ), acetyl-LDL (▽), collagen IV (◇), RNAse (□). LDL nmoles were calculated on the basis of the molecular weight of apoprotein B.

C: Reaction with model AGEs. Glucose-derived AGE-lysine (●), G6P-derived AGE-lysine (♦), FFI-HA (○), pyrraline (▲), AFGP (▼), carboxymethyllysine (■), pentosidine (□), deoxypropylaminofructose (Δ), and deoxymorpholinofructose (▽). AGE-lysine products were added as nmoles of lysine equivalents;

FIG. 3 is the kinetic relationship between the formation of AGE-associated fluorescence and antibody reactive material. BSA (50 mg/ml) was incubated with glucose (0.5M) as described in Materials and Methods. Aliquots were sampled at indicate times, dialyzed to remove unbound material, and assayed for fluorescence ($\lambda_{excitation}$=370 nm, $\lambda_{emission}$=440 nm) and AGE content by ELISA;

FIG. 4 is the inhibition of the formation of antibody-reactive AGEs by aminoguanidine. BSA (100 mg/ml) incubated with glucose (100 mM) for 21 days at 37° (○). BSA (100 mg/ml) incubated with glucose (100 mM) in the presence of 100 mM aminoguanidine for 21 days at 37° (●). Buffer conditions were as described in Materials and Methods;

FIG. 5 is the determination of AGE-collagen in experimental rats. Diabetes was induced in Lewis rats with either alloxan or streptozotocin as described in Materials and Methods. At 16 week intervals, 6 animals were sacrificed and the aortic collagen analyzed for hydroxyproline, fluorescence, and AGE-content by ELISA. Values are expressed per mg of hydroxyproline.

(A): Relative fluorescence measured at $\lambda_{excitation}$=370 nm and $\lambda_{emission}$=440 nm).

(B): Collagen-bound AGEs measured by ELISA. Control rats (○), rats with alloxan-induced diabetes (▲), and rats with streptozotocin-induced diabetes (▼). Each value shown is the mean of six experimental animals;

FIG. 6 is the determination of human rerum AGE levels. NL: Normal individuals (n=12). DM: Diabetic individuals (n=21). DM+HD: Diabetic individuals on hemodialysis (n=16). Error bars show the S.E.M. P<0.001 for DM vs. NL. P<0.001 for DM+HD vs. DM.;

FIG. 7 is a graph comparing Hb-AGE and HbA_{1c} levels in red blood cells of normal and diabetic patients and thereby depicting the correlation between the two (normal patients=■) (diabetic patients=□);

FIG. 8 comprises two bar graphs that show and compare the mean of Hb-AGE (A) and HbA_{1c} (B) levels in normal and diabetic patients;

FIG. 9 is a graph depicting the results of the ELISA of Hb-AGE levels measured in 23 diabetic individuals (DM) and 9 non-diabetic, normoglycemic individuals (NL). NL: 4.3±0.3 AGE Units/mg Hb, DM: 7.7±0.6 AGE Units/mg Hb. (Mean±S.E.). Each value represents the mean of triplicate determinations, assayed at 3–4 hemoglobin dilutions to ensure that measured values fell within the linear range of the ELISA standard curve. AGE units are calculated relative to an AGE-albumin standard synthesized and analyzed for AGE content as described.

FIG. 10 graphically shows a correlation between levels of Hb-AGE and HbA_{1c} for nine normoglycemic (○) and 23 diabetic (●) individuals. HbA_{1c} was measured by HPLC.

FIG. 11 is a graph of Hb-AGE formation in vitro. Human hemoglobin (50 mg/ml) (Sigma Chemical Co.) was incubated at 37° C. in 0.4M $NaPO_4$ buffer (pH 7.4) containing 0 mM glucose (○), 5 mM glucose (●), 20 mM glucose (▲), or 20 mM glucose and 50 mM aminoguanidine (♦). Aliquots containing hemoglobin and 20 mM glucose were reduced with a 200-fold molar excess of sodium borohydride prior to ELISA (▽). Aminoguanidine (50 mM) also inhibited (>95%) Hb-AGE formation in the 5 mM glucose condition (data not shown). Aminoguanidine (50 mM) did not inhibit the detection of Hb-AGE when added to glucose/hemoglobin incubations 1 hour prior to ELISA analysis (data not shown). All incubations were performed after sterile filtration. One ml samples were removed at the indicated time points and dialyzed against phosphate-buffered saline prior to ELISA analysis.

FIG. 12 is a determination of Hb-AGE and HbA_{1c} levels in 18 diabetic patients before (■) and after (▥) 28 days of aminoguanidine therapy. Hb-AGE: 13.8±0.8 U AGE/mg Hb->10.0±0.9 U AGE/mg Hb; HbA_{1c}: 10.1%±0.8%→9.2%±0.8% (Mean±S.E.).

DETAILED DESCRIPTION OF THE INVENTION

Numerous abbreviations are used herein to simplify the terminology used, and to facilitate a better understanding of the invention. The following abbreviations are representative.

As used herein, the terms "AGE" and "AGEs" are used as appropriate to refer to advanced glycosylation endproducts which are in the form of intermediates and stable compounds which are produced in vivo and in vitro by the reaction of reducing sugars with protein amino groups. AGEs therefore encompass intermediates as well as stable endproducts that are implicated in the structural and functional alteration of proteins seen during aging. For example, AGEs are recognized to react with free polypeptide amino groups, which leads to protein crosslinking. Additionally, such AGEs are observed in elevated levels in circulation and in tissues in certain diseases, e.g. diabetes mellitus.

When the designations "AGE-RNAse", "AGE-Hb", "AGE-BSA", "AGE-HSA", "AGE-albumin", "AGE-collagen" and "AGE-LDL" are used, each refers to the advanced glycosylation endproducts which are formed upon chemical reaction of the substrates RNAse, Hb, BSA, HSA, albumin, collagen and LDL, respectively with one or more reducing sugars. Thus, AGE-RNAse refers to the advanced glycosylation endproducts of the reaction between bovine ribonuclease and a reducing sugar.

Albumin, when recited generically, refers to any specie from which it was obtained, e.g., human, bovine, etc.

BSA refers to bovine serum albumin.

HSA refers to human serum albumin.

RNAse refers to ribonuclease generally, and where appropriate, to bovine pancreatic ribonuclease in particular.

Collagen is used in the conventional sense to refer to any type of collagen and derived from any appropriate source. When a specific type of collagen was used, such as in the

example, the particular type is noted. However, it is recognized that alternative collagen types can also be used.

LDL is also used in the conventional sense to refer to low density lipoprotein.

FFI-BSA refers to a model AGE-protein produced by incubating 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole hexanoic acid with bovine serum albumin and coupling the reactive compounds with dicyclohexylcarbodiimide. Formaldehyde-BSA, maleyl-BSA and acetyl-LDL all refer to modified proteins produced as described below.

The present invention relates to the measurement of certain in vivo-generated AGEs and particularly Hb-AGE, serum AGE peptides and urinary AGE peptides, and to the diagnostic and therapeutic applications to which such measurement may be put. The use of Hb-AGE as a marker facilitates the long-term measurement of blood sugar levels and the consequent ability to monitor glycemic conditions such as exist in diabetes mellitus. As the data presented herein reveals, Hb-AGE in contrast to the known determinant HbA_{1c}, decreases in response to the administration of the in vivo glycation inhibitor aminoguanidine and thus represents a clinically accurate time-integrated parameter of recent blood sugar levels that possesses the added dimension of use for both diagnostic and therapeutic purposes.

Particularly, the present invention takes advantage of a competitive ELISA developed with the anti-AGE antibodies that form a part hereof and that are also comprehensively presented in parent application Ser. No. 07/811,579, abandoned, and comprises a quick and effective diagnostic method. As shown in FIG. 9, Hb-AGE represents a stable and reliable glucose-derived rearranged Amadori product around which a diagnostic protocol may be developed and carried out. Accordingly, the invention extends to a method for the long term monitoring of in vivo glycemic conditions and the concomitant assessment of the efficacy of any control measures that may be implemented.

The present invention also extends to the measurement of other AGEs and particularly serum and urinary AGE-peptides. Serum and urinary AGE-peptides, like Hb-AGE, represent circulating markers of AGE accumulation that reflect the onset and extent of pathologies and other dysfunctions where such accumulation is a characteristic. Thus, those age-related and diabetic conditions where increased levels of AGEs have been observed, such as, for example, atherosclerosis, cataracts and diabetic nephropathy, may be monitored and assessed over the long term by the measurement of these AGEs, particularly by resort to the diagnostic methods disclosed herein.

Likewise, the methods including Hb-AGE or the noted AGE-peptides as markers or determinants may be used as a drug discovery assay, for identification of drugs or other modalities that may interact with these AGEs. In the instance of Hb-AGE, it may be possible to discover agents that may control either the concentration of blood glucose or the formation of Hb-AGE itself. An assay or test kit in such instance would include the reagents set forth for such kits presented later on herein, with Hb-AGE as the ligand or binding partner. A similar assay or kit may be prepared with the noted AGE peptides serving in like capacity to Hb-AGE.

The investigation of AGE formation in vivo has been hampered by a lack of specific assay methods and by the general inaccessibility of tissue AGEs to in vivo analysis. The realization that Amadori products such as HbA_{1c} are AGE precursors led to a consideration that hemoglobin might also acquire AGE modifications that could be measured with recently developed ELISA techniques.

Hb-AGE has been determined to account for about 0.42% of circulating human hemoglobin. This fraction increases to approximately 0.75% in patients with diabetes-induced hyperglycemia. Of significance, diabetic patients treated for 28 days with aminoguanidine, an inhibitor of AGE formation in vivo, showed significantly decreased levels of Hb-AGE at the end of the treatment period.

The tissue and end organ damage caused by advanced glycosylation accumulates over a period of months to years. Diabetic complications progress over a similar duration.

As stated above, a particularly preferred aspect of the present invention is the use of Hb-AGE as an indicator of the extent of control of blood glucose in diabetes patients. Taking into account the normal Hb-AGE levels and the reaction time over which Hb-AGE is formed, the level of Hb-AGE present in the blood can be predictive of diseases and underlying pathology where the level of Hb-AGE is above normal values.

Similarly, serum peptide-AGEs can be used as an indicator that reflects glomerular filtration rate (GFR) and kidney damage. Urinary AGE-peptides may be used as an indicator to measure the turnover in tissue proteins, and more particularly, tissue-AGE proteins.

In both the Hb-AGE and the serum AGE-peptide assays, the blood sample is drawn and a separation procedure can be used. The cellular blood components can be separated from the serum, and in the Hb-AGE assay, the hemoglobin can be extracted from the red blood cells. The serum level of AGE-peptides and the presence or extent of Hb-AGEs present can then be evaluated.

By conducting both tests with a single blood sample, a broader time frame at which blood glucose levels become uncontrolled can be estimated, e.g., a 60 day range predictable by Hb-AGE that extends to the periods before, during or after the 3–4 week time frame which is predicted by $Hb\text{-}A_{1c}$. If desired, the analyses of Hb-AGE and serum AGE-peptides can also be run together with a glucose level determination in blood or urine, a glucose tolerance test, and other tests useful for assessing diabetes control including the measurement of urinary AGE-peptides, to give a complete patient profile.

Another aspect of the invention addresses advanced glycosylation endproducts which can be detected in the urine. Proteins, including peptides, are excreted in the urine in low levels in normal individuals, and in perhaps elevated levels in diseased individuals. The presence and/or level of urinary AGE-peptides reflective of the turnover of tissue AGEs can be determined, correlated to and predictive of particular diseases or conditions. For example, the quantity of peptides found in normal urine usually ranges from about 25 to 50 mg per day and is comprised of microproteins, with properties which are quite different from the predominant blood proteins, albumin and globulin.

The presence of proteins in the urine may be a symptom of numerous diseases or conditions reflective of a net catabolic state as would exist when the host or patient is undergoing invasion as by infection. Under such circumstances, the host mobilizes against the invasive stimulus by the secretion of numerous factors such as cytokines that suspend the anabolic energy storage activity and cellular repair activities and promote instead the catabolic depletion of energy stores and the recruitment of leukocytes and other factors to fight and neutralize the stimulus. As this activity results in a corresponding elevation in tissue protein levels, and corresponding levels of tissue-AGEs, the measurement of urinary AGE-peptides provides

yet another index of possible invasive activity in the host, such as cachexia and shock. Thus, one can measure the presence or level of AGE-peptides in urine, and correlate this level to a standard. In normal individuals, the normal level may be at or close to zero. In diabetic patients or in patients experiencing infection or other trauma, the normal level of AGE-peptides may be significantly greater. Thus, the advancement or worsening of diabetes prior to the onset of renal complications, or the presence of infection could be detected by detecting increases in urine levels of AGE-peptide.

Likewise, one may be able to detect individuals who glycosylate proteins faster than normal. In this instance, one would determine the level of AGE-peptides in the urine as a result of a specific challenge, e.g., with glucose, a compound which induces AGE formation or release by the proteins of the body, or the rate at which urinary peptide-AGE levels increase after cessation of the administration of an AGE inhibiting compound, e.g., aminoguanidine. A full clinical picture will therefore become apparent.

The present invention also relates to detecting the presence or level of such AGE-peptides in serum. This may take into account the extent of AGE accumulation and reaction with extracellular and cellular blood proteins, protein fragments and peptides (shorter chain amino acids, e.g., up to about 50 amino acids in length) found in the circulatory system. These can be evaluated for the presences or level of AGEs, the extent of advanced glycation determined, and compared to a standard, e.g., normal peptide glycation levels. For example, if one detects an elevated level of AGE-peptides in the blood, one may correlate this to the extent of kidney damage sustained by the patient.

Amadori products are slowly reversible and are believed to attain equilibrium over a 3–4 week period. AGEs, in contrast, remain irreversibly attached to proteins and continue to accumulate over the lifespan of the protein. The utility of Hb-AGE measurements as a long term in vivo marker of advanced glycosylation can thereby be appreciated.

As stated earlier, the invention also comprises the identification of an antiserum and corresponding antibodies that recognize and bind to in vivo-formed advanced glycosylation endproducts. As demonstrated in the examples presented later on herein, polyclonal AGE-ribonuclease antiserum has been prepared and used in competitive ELISA systems to study the specificity of this antiserum for in vitro- and in vivo-derived products. The resulting antiserum recognized and bound to in vivo AGEs in diabetic tissue and serum known to contain abnormally elevated levels of AGEs, leading to the conclusion that a common epitope exists among them. By contrast, each of the model, synthetic AGEs which were synthesized and tested failed to react with the polyclonal antiserum, although other in vitro-formed AGEs are reactive.

Examples of proteins and protein-containing substances suitable for incubation-type reactions with the reducing sugars include, for example, RNAse, hemoglobin, collagen, BSA and HSA, each of which can be incubated with, e.g., glucose, glucose-6-phosphate ("G6P"), fructose or ribose to produce a suitable AGE immunogen for inducing the formation of anti-AGE antibodies.

Such anti-AGE antibodies can also be used in the treatment of patients to reduce the level of circulating AGEs or AGEs which may be present. in abnormally elevated levels in certain tissues, e.g., pancreas, liver, kidney or brain.

Additionally, it is within the scope of the invention described herein to utilize the anti-AGE antibodies for the design, screening and/or potentiation of drugs or compounds which are useful for treating elevated levels of AGEs in vivo. In this connection, the anti-AGE antibodies may be used to purify proteins that have been specially cultivated or produced for use as therapeutic agents. As stated earlier, the therapeutic use of such proteins is increasing in prominence and importance, and such proteins like other host cells, are susceptible to glycation and the formation of AGEs. As such AGEs are particularly clinically active, it is desirable to limit their introduction into a host during therapy. As a consequence, the present invention includes a method for purification of batches of such proteins by bringing them into contact with, for example, a quantity of anti-AGE antibodies immobilized on a suitable substrate. In this way the glycosylated proteins could be separated from the rest of the batch by conventional procedures. The substrate could be refreshed or replaced periodically in the instance of a commercial process, so that a continuous circulation of protein material past the substrate and subsequent separation of the protein-AGE component could be conducted. Naturally, the foregoing scheme is presented for purposes of illustration only, and is capable of various modifications in design and execution within the skill of the art and the scope of the invention.

The invention also includes methods for measuring protein aging both in plants and in animals, by assaying the presence, amount, location and effect of such advanced glycosylation endproducts utilizing the anti-AGE antibodies. By reacting anti-AGE antibodies with samples of products suspected of containing AGEs, plant matter and animal food samples can be evaluated to assess food spoilage and the degradation of the proteinaceous materials so affected, while the assays of animals, including body fluids such as blood, plasma and urine, tissue samples, and biomolecules such as DNA, that are capable of undergoing advanced glycosylation, assist in the detection of pathology or other systemic dysfunction.

Further, such assay may be employed to assess the extent of degradation of proteins that have been cultivated, harvested or otherwise recombinantly prepared for therapeutic use, to determine whether and/or to what extent such materials have become glycosylated. This assay could be used alone or in conjunction with a purification procedure, so that the determination that the protein material has developed a predetermined threshold level of glycosylation would signal the need for performing a purification procedure such as that described above, on a batchwise or continuous basis.

The assay methods of the invention comprise the performance of several assay protocols, involving the anti-AGE antibodies described herein whether labeled or not, the analyte, and/or a ligand, one or more binding partners to the antibody, and binding partners thereto as applicable.

The term "binding partners" is used in the general sense to refer to components used in the assays which recognize and/or bind to each other. Thus, an anti-AGE antibody and the AGEs recognized by the antibody are considered binding partners.

The term "binding partners" also includes ligands useful in the present invention, such as AGE derivatives that bind to AGE binding partners. These ligands may be detected either singly and directly, or in combination with a second detecting partner such as avidin or biotin. Suitable synthesized AGE derivatives are selected from the reaction products of reducing sugars, such as glucose, glucose-6-phosphate (G6P), fructose and ribose and peptides, proteins and other biochemicals such as BSA, avidin, biotin derivatives, and enzymes such as alkaline phosphatase.

Useful with this invention are enzymes and other carriers that have undergone advanced glycosylation. These AGE-enzymes may serve as the preferred labelled ligands in the assays of the present invention. Other suitable ligands may include the reaction product of the reducing sugars directly with carriers capable of undergoing advanced glycosylation. Suitable carriers may be comprised of a material selected from carbohydrates, proteins, synthetic polypeptides, lipids, bio-compatible natural and synthetic resins, and mixtures thereof.

The assays of the invention may follow formats wherein either the ligand or the binding partner are bound to a substrate. Likewise, the assays include the use of labels which may be selected from radioactive elements, enzymes and chemicals that fluoresce.

The present methods have particular therapeutic relevance in that means for the detection and evaluation of the condition of a broad spectrum of organ systems are provided. The Maillard process acutely affects several of the significant protein masses in the body, among them collagen, elastin, lens proteins, and the kidney glomerular basement membranes. These proteins deteriorate both with age (hence the application of the term "protein aging") and as a result of prolonged exposure to elevated blood sugar levels and AGE formation, the latter in turn frequently due to pathology.

In this manner, the location and relative concentrations of advanced glycosylation endproducts in the body can be identified. Moreover, by assaying different organ specimens, an AGE "patient profile" can be obtained, i.e., the location and relative concentration of AGEs in the patient can be identified. This technique is particularly useful in identifying abnormal concentrations of advanced glycosylation endproducts, such as in atheromatous plaques. In such manner, the location of future systemic malfunctions can be identified.

Accordingly, the present assay method broadly comprises the steps of:

A. preparing at least one sample suspected of containing said advanced glycosylation endproducts;

B. preparing at least one anti-AGE antibody directed to said samples, wherein the anti-AGE antibody is reactive with in vivo-produced advanced glycosylation endproducts and has the following characteristics:

i. it reacts with an immunological epitope common to said in vivo-formed advanced glycosylation endproducts;

ii. it is cross reactive with advanced glycosylation endproducts formed in vitro; and

iii. it is not cross reactive with the following advanced glycosylation endproducts however formed: FFI, AFGP, pyrraline, carboxymethyllysine, and pentosidine;

C. placing a detectible label on a material selected from the group consisting of said sample, a ligand to said anti-AGE antibody and said anti-AGE antibody;

D. placing the labeled material from Step C in contact with a material selected from the group consisting of the material from Step C that is not labeled; and

E. evaluating the resulting sample of Step D for the extent of binding of said labeled material.

Suitable analytes which can be evaluated may be selected from, e.g., plant matter, edible animal matter, blood, plasma, urine, cerebrospinal fluid, lymphatic fluid, and tissue; and certain synthesized compounds, individually and bound to carrier proteins such as the protein albumin. The analyte may also comprise a synthetically derived advanced glycosylation endproduct which is prepared, for example, by the reaction of a protein or another macromolecule with a reducing sugar. This reaction product can be used alone or combined with a carrier.

The carrier may be selected from the group consisting of carbohydrates, proteins, synthetic polypeptides, nucleic acids, lipids, bio-compatible natural and synthetic resins, antigens and mixtures thereof.

The anti-AGE antibodies described herein can be used both to diagnose the degradative effects of advanced glycosylation of proteins in plants, edible animal matter, and the like, and to detect the adverse effects of the buildup of advanced glycosylation endproducts. Such conditions as age- or diabetes-related hardening of the arteries, skin wrinkling, arterial blockage, and diabetic, retinal and renal damage in animals all result from the excessive buildup or trapping that occurs as advanced glycosylation endproducts increase in quantity. Therefore, the diagnostic methods of the present invention seeks to avert pathologies caused at least in part by the accumulation of advanced glycosylation endproducts in the body by monitoring the amount and location of such AGEs.

Using the present invention, one can assess and/or detect the presence of stimthe presence of stimulated, spontaneous, or idiopathic pathological states in mammals, by measuring the corresponding presence of advanced glycosylation endproducts. More particularly, the presence or amount of the AGEs may be followed directly by assay techniques such as those discussed herein, through the use of an appropriately labeled quantity of the present anti-AGE antibodies or at least one of their binding partners, as set forth herein. Alternately, AGEs defining epitopes reactive with the present anti-AGE antibodies, could be synthesized and used to raise binding partners (or antagonists to such binding partners) that could in turn, be labeled and introduced into a medium to test for the presence and amount of AGEs therein, and to thereby assess the state of the host from which the medium was drawn.

Thus, both AGE receptors and any binding partners thereto that may be prepared, are capable of use in connection with various diagnostic techniques, including immunoassays, such as a radioimmunoassay, using for example, a receptor or other ligand to an AGE that may either be unlabeled or if labeled, then by either radioactive addition, reduction with sodium borohydride, or radioiodination.

The general assay procedures and their application are all familiar to those skilled in the art and are presented herein as illustrative and not restrictive of the procedures that may be utilized within the scope of the present invention.

The advanced glycosylation endproduct forms complexes with one or more of the binding partners, and one member of the complex may be labeled with a detectable label. The fact that a complex has formed and, if desired, the amount thereof, can be determined by applicable detection methods, e.g., IgG recognition and reaction with the complexes formed.

Suitable radioactive elements may be selected from the group consisting of ^{3}H, ^{14}C, ^{32}P, ^{35}S, ^{36}Cl, ^{51}Cr, ^{57}Cr ^{58}Co, ^{59}Fe, ^{90}Y, ^{125}I, ^{131}I, and ^{186}Re. In the instance where a radioactive label, such as prepared with one of the above isotopes is used, known currently available counting procedures may be utilized.

In the instance where the label is an enzyme, detection may be accomplished by any of the presently utilized colorimetric, chemiluminescent, spectrophotometric,

fluorospectro-photometric, thermometric, amperometric or gasometric techniques known in the art. The enzyme may be conjugated to the advanced glycosylation endproducts, their binding partners or carrier molecules by reaction with bridging molecules such as carbodiimides, diisocyanates, glutaraldehyde and the like. Also, and in a particular embodiment of the present invention, the enzymes themselves may be modified into advanced glycosylation endproducts by reaction with sugars as set forth herein.

Many enzymes which can be used in these procedures are well known and can be utilized. The preferred enzymes for detection are peroxidase, β-glucuronidase, β-D-glucosidase, β-D-galactosidase, urease, glucose oxidase plus peroxidase, hexokinase plus GPDase, RNAse, glucose oxidase plus alkaline phosphatase, NAD oxidoreductase plus luciferase, phosphofructokinase plus phosphoenol pyruvate carboxylase, aspartate aminotransferase plus phosphoenol pyruvate decarboxylase, and alkaline phosphatase. U.S. Pat. Nos. 3,654,090; 3,850,752; and 4,016,043 are referred to by way of example for their disclosure of alternative labeling materials and methods.

A number of fluorescent materials are also known which can be utilized as labels. These include, for example, fluorescein, rhodamine and auramine. A particularly preferred detecting material includes one or more fluorescent labels on anti-rabbit antibodies prepared in goats and conjugated with fluorescein through an isothiocyanate.

One assay format contemplates a bound antibody to which are added the ligand and the analyte. The resulting substrate is then washed after which detection proceeds by the measurement of the amount of analyte bound to the antibody. A second format employs a bound ligand to which the antibody and the analyte are added. Both of these formats are based on a competitive reaction with the analyte, while a third format comprises a direct binding reaction between the bound analyte and the antibody. In the latter two formats, the extent of binding of the antibody is measured by a direct label or a labeled binding partner.

All of the protocols disclosed herein may be applied to the qualitative and quantitative determination of advanced glycosylation endproducts and to the concomitant diagnosis and surveillance of pathologies in which the accretion of advanced glycosylation endproducts is implicated. Such conditions as diabetes and the conditions associated with aging, such as atherosclerosis and skin wrinkling represent non-limiting examples, and accordingly methods for diagnosing and monitoring these conditions are included within the scope of the present invention.

The present invention also includes assay and test kits for the qualitative and/or quantitative analysis of the extent of the presence of advanced glycosylation endproducts. Such assay systems and test kits may comprise a labeled component prepared, e.g., by one of the radioactive and/or enzymatic techniques discussed herein, coupling a label to an anti-AGE antibody or a binding partner as listed herein; and one or more additional immunochemical reagents, one of which may be a free or immobilized ligand, capable either of binding with the labeled component, its binding partner, one of the components to be determined or their binding partner(s). One of the components of the kits described herein is typically an anti-AGE antibody in labeled or non-labeled form.

In a further embodiment of this invention, commercial test kits suitable for use in one instance by food technologists, and in other instances by medical specialists may be prepared to determine the presence or absence of advanced glycosylation endproducts. As stated earlier, the kits may be used to measure the presence of advanced glycosylation endproducts on recombinant or other purified proteins, and particularly those destined for therapeutic use, to assay them in a first instance, and in a second instance, to assist in their further purification.

In accordance with the testing techniques discussed above, one class of such kits will contain at least labeled AGE, or its binding partner as stated above, and directions, of course, depending upon the method selected, e.g., "competitive", "sandwich", "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Accordingly, a preferred test kit may be prepared for the demonstration of the presence, quantity or activity of AGEs, comprising:

(a) a predetermined amount of at least one labeled immunochemically reactive component obtained by the direct or indirect attachment of the anti-AGE antibodies of the present invention or a specific binding partner thereto, to a detectable label;

(b) other reagents; and

(c) directions for use of said kit.

More specifically, the preferred diagnostic test kit may comprise:

(a) a known amount of a binding partner to an anti-AGE antibody as described above, or a ligand thereof, generally bound to a solid phase to form an immunosorbent, or in the alternative, bound to a suitable tag, or plural such end products, etc. (or their binding partners) one of each;

(b) if necessary, other reagents; and

(c) directions for use of said test kit.

In a further variation, the preferred test kit may comprise:

(a) a labeled component which has been obtained by coupling the binding partner of the anti-AGE antibody to a detectable label;

(b) one or more additional immunochemical reagents of which at least one reagent is a ligand or an immobilized ligand, which ligand is selected from the group consisting of:

(i) a ligand capable of binding with the labeled component (a);

(ii) a ligand capable of binding with a binding partner of the labeled component (a);

(iii) a ligand capable of binding with at least one of the component(s) to be determined; and

(iv) a ligand capable of binding with at least one of the binding partners of at least one of the component(s) to be determined; and

(c) directions for the performance of a protocol for the detection and/or determination of one or more components of an immunochemical reaction between the anti-AGE antibody and a specific binding partner thereto.

The present invention extends to the production of the anti-AGE antibodies in a mammal, and an antiserum containing said antibodies. For example and as illustrated herein, a mammal can be immunized with the incubation product of a reducing sugar and any protein which contains free amino groups, and which is subject to Amadori rearrangement, thus yielding AGEs.

As used herein, the terms "immunized" and "hyperimmunized" refer to the specific immunization protocol also described in detail later on herein, which is used to elicit the antibody response that yields the antiserum and antibodies of the present invention. The protein or the reaction product

of the protein described above incubated with one or more reducing sugars can be used. Typically about four primary doses of the immunogen are administered to an immunocompetent mammal in an amount effective for inducing the formation of anti-AGE antibodies. Booster immunization doses may also be administered as appropriate.

The immunization of the host mammal with the protein itself or with AGEs derived from the AGE-protein, e.g., AGE-RNAse, produces antibodies which are reactive to AGEs derived from the immunogen itself or AGEs which are derived from other proteins. For example, the administration of AGE-RNAse produces polyclonal anti-AGE antibodies which react with AGE-RNAse, AGE-hemoglobin, AGE-BSA, AGE-HSA, AGE-LDL and AGE-collagen IV, but not with unmodified RNAse, BSA or HSA.

The antibodies raised as described above were also generally unreactive with model AGEs produced synthetically through procedures involving chemical hydrolysis or reduction, such as the model AGEs FFI, AFGP, pyrraline, carboxymethyllysine and pentosidine. None of these model compounds was recognized by the anti-AGE antibodies raised.

The invention described herein takes advantage of the epitope which is present on these in vivo-derived AGEs and on in vitro-generated AGEs produced without specific chemical hydrolysis or reductive conditions. The epitope can be exploited in numerous processes for detecting AGEs on in vivo-derived as well as in vitro-derived material. For example, the binding affinity of anti-AGE antibodies can be used in non-competitive and competitive ELISA assays, as well as in other protocols which utilize different immunoassay configurations.

Ribonuclease modified by long-term incubation with glucose was found to be a suitable immunogen for the production of high-titre, anti-AGE antibodies against a variety of AGE-modified proteins. Advanced glycosylation endproducts prepared with glucose showed the greatest inhibition, followed by AGEs prepared with G6P and fructose. Both G6P and fructose react with proteins at a faster rate than glucose to produce brown, fluorescent AGEs.

Different glycosylating sugars such as glucose, G6P, and fructose produce antigenically cross-reactive epitopes when incubated with protein in vitro. In contrast, the purification of particular products from in vitro incubation mixtures of polypeptides or amines with glucose (FFI, AFGP, pyrraline) or from tissue glycosylated in vivo (pentosidine) resulted in compounds with no demonstrable cross-reactivity with anti-(AGE-RNAse) antiserum. This suggests that the model AGEs which have been described thus far are either antigenically minor products, or that the purification procedures which are typically used for isolating AGEs from a sample resulted in structural alterations, such that reactivity with anti-AGE antibodies is essentially eliminated.

In vitro time course studies revealed that the characteristic fluorescence of advanced glycosylation precedes the development of the anti-AGE reactive moieties. Thus, the anti-AGE antiserum appears to be most reactive with "late" advanced glycosylation endproduct(s) which form after fluorophore formation.

The ligands useful in the present invention are preferably in vivo-generated AGEs that bind to AGE binding partners. These ligands may be detected alone or in combination with a second detecting partner such as avidin and/or biotin. Suitable ligands when these signal amplifiers are used can be selected from the reaction products of reducing sugars such as glucose, G6P, fructose, ribose and the like with peptides, proteins and other biochemicals such as BSA, avidin, biotin, and enzymes such as alkaline phosphatase.

As discussed earlier herein, the invention extends to monoclonal anti-AGE antibodies which are capable of preparation by hybridoma techniques, utilizing, for example, fused mouse spleen lymphocytes and myeloma cells. Immortal, antibody-producing cell lines can also be created by techniques other than fusion, such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus.

Specific polyclonal antibodies can be raised in different preferred host species. Naturally, these antibodies are merely illustrative of antibody preparations that may be made in accordance with the present invention.

Specific protocols are illustrated below as necessary. The protocols disclosed herein may be applied to the qualitative and quantitative determination of AGEs and to the concomitant diagnosis and surveillance of pathologies in which the accretion of AGEs is implicated. Conditions such as diabetes and those associated with aging, such as atherosclerosis and skin wrinkling, represent non-limiting examples. Accordingly, methods for diagnosing and monitoring these conditions are included within the scope of the present invention.

The biochemical and biological reagents which are recited below are available commercially or prepared in accordance with recognized protocols. All publications cited herein are hereby incorporated by reference.

MATERIALS AND METHODS

Reagents

The reagents used in the assays described below were obtained or prepared as follows:

Bovine pancreatic ribonuclease ("RNAse"), bovine serum albumin ("BSA"), human serum albumin ("HSA"), collagen Type 4, collagenase, glucose, glucose-6-phosphate ("G6P"), fructose, ribose, and sodium borohydride were obtained from Sigma Chemical Corp. (St. Louis, Mo.). AGE-hemoglobin was prepared by isolating red blood cells, hemolyzing them with toluene and treating a sample of the red cell hemolysate with trichloroacetic acid (TCA). Specifically, a 50–100 µl sample of RBC hemolysate was prepared and 3 ml of water and 1 ml of 24% (wt/vol) TCA were added. The mixture was agitated, and thereafter centrifuged for 30 minutes at 3000 rpm. The resulting supernatant was aspirated, 150 µl NaOH was then added, after which water was added to a total volume of 0.5 ml. This material was then diluted from 1:2 to 1:200 with 0.3M KH_2PO_4 pH 7.4, and was prepared for performance of the assay.

AGE-albumin was prepared by incubating albumin (50 mg/ml) with 0.5M glucose, G6P or fructose in 0.2M $NaPO_4$ buffer (pH 7.4) for 60 days.

AGE-collagen was synthesized by incubating collagen (5 mg/ml) with 0.5M glucose in 0.2M $NaPO_4$ buffer (pH 7.4) for 21 days.

AGE-RNAse was prepared by incubating RNAse (25 mg/ml) with 1M glucose in 0.2M $NaPO_4$ buffer (pH 7.4) for 90 days.

FFI-BSA was prepared by coupling FFI-hexanoic acid to BSA with carbodiimide.

Formaldehyde-BSA, maleyl-BSA and acetyl-LDL were synthesized in accordance with the procedures described in Horiuchi, S. et al., *J. BIOL. CHEM.*, 4:260, 432, 438 (1985); Takata, K. et al., *BIOCHIM. BIOPHYS. ACTA.*, 94:273–280 (1989); and Goldstein, J. L. et al., *PROC. NATL. ACAD. SCI. USA*, 77:333–337 (1979).

AGE-LDL was prepared according to a protocol in which spontaneous oxidation is minimized, according to

Kirstein, M. et al, *PROC. NATL. ACAD. SCI. USA*, 87:9010–9014 (1990).

AGE-BSA was reduced with sodium borohydride as described in Flückiger, R. et al, *METHODS ENZYMOL.*, 106:77–87 (1984). Any unreacted borohydride was removed by dialysis against PBS.

For aminoguanidine inhibition, BSA (100 mg/ml) was incubated with 100 mM glucose and 100 mM aminoguanidine hydrochloride (Aldrich Chemical Co., Milwaukee, Wis.) in 0.2M $NaPO_4$ buffer (pH 7.4) for 21 days at 37° C. Samples were dialyzed against PBS prior to analysis.

Lysine-derived AGEs were prepared by incubating 1M glucose-6-phosphate or 1M glucose with 50 mM L-lysine in 0.2M sodium phosphate buffer (pH 7.4) 10 days at 37° C.

1-deoxy-1-morpholino-D-fructose was obtained from Sigma Chemicals, Inc. and 1-deoxy-1-propylamino-D-fructose was prepared from an α-D-glucose and N-propylamine according to Mitchel, F. et al., *CHEM. BER.*, 92:2836–2840 (1959). 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole) was synthesized from an aqueous mixture of furylglyoxal and 6-aminohexanoic acid and purified by medium pressure chromatography on silica gel.

1-alkyl-2-formyl-3,4-diglycosyl-pyrrole (AFGP) was prepared by incubating glucose with 6-aminohexanoic acid and sodium sulfite for 26 days at 37° C. followed by chromatography on Dowex AG 1×4 anion exchange resin and HPLC.

OTHER METHODS

The anti-AGE antibody and its reactivity with in vivo-derived AGEs, and its non-reactivity with AGEs synthesized chemically by methods involving hydrolysis or reduction were evaluated. Protein concentrations were determined in accordance with Bradford, M., *ANAL. BIOCHEM.*, 72:248–252 (1976), and BSA was utilized as a standard. Ribonuclease and AGE-RNAse protein amounts were determined additionally by SDS-PAGE and comparison of Coomassie Blue stained bands with RNAse standards. Hydroxyproline content was determined according to Edward, C. et al., *CLIN. CHIM. ACTA*, 104:161–167 (1980). Collagen AGE-specific fluorescence determinations were performed by measuring emissions at 440 nm upon excitation at 370 nm using an LS-3B fluorescence spectrometer (Perkin-Elmer, Norwalk, Conn.).

A preparation of control albumin was also incubated under the same conditions described above without sugar. All incubations were performed under sterile conditions in the dark, and at 37° C. After incubation, unbound material was removed by extensive dialysis against phosphate buffered saline (PBS) or by gel filtration over Sephadex G-10 (Pharmacia, Uppsala, Sweden).

A single standard AGE-BSA preparation (1 mM AGE-BSA=12 A_{350}) was used as a reference. Fluorescence intensity standards were used to calibrate and monitor the performance of the instrument. Fluorescence values of test substances were measured at a protein concentration of 1 mg/ml and expressed as percent relative fluorescence compared to the AGE-BSA standard.

PRODUCTION OF ANTI-AGE ANTIBODIES

Previous efforts to raise antisera against native AGEs generally have been unsuccessful or have failed to detect AGEs which occur in vivo.

To induce the formation of anti-AGE antibodies, synthesized AGEs were produced in vitro as described above without resort to hydrolytic or chemical reduction reactions. The protein was typically incubated with a reducing sugar to form AGEs.

Glucose was preferably used as the glycosylating agent in vitro because it is the major circulating sugar and it produces AGEs in vitro which closely resemble the AGEs which are formed in vivo. RNAse was selected as the target protein for advanced glycosylation because RNAse readily forms AGEs and AGE-mediated intermolecular crosslinks.

Two female New Zealand White rabbits received four primary immunizations and one booster immunization of RNAse or AGE-RNAse emulsified in Freund's complete adjuvant following a protocol for post-translationally modified proteins in accordance with R. Bucala, et al., *Mol. Immunol.*, 20:1289–1292 (1983) ("hyperimmunization protocol") and as follows. Accordingly, each rabbit received four intradermal injections over the back (200 μg each) and one injection in each hind quarter (100 μg each). This procedure was repeated at weekly intervals for six weeks. After a two week rest, the rabbits received a booster injection of 1 mg of antigen in Freund's incomplete adjuvant. The amimals were bled on the tenth day after this injection. Antibody response was monitored weekly by Ouchterlony double diffusion and by non-competitive ELISA.

Using the hyperimmunization protocol described above, high titre polyclonal rabbit anti-serum against RNAse (the carrier protein) was obtained. The anti-RNAse titre was determined to be greater than 10^{-5} in a non-competitive ELISA. The following absorbed antigens were tested: RNAse, glucose-derived AGE-RNAse, BSA, glucose-derived AGE-BSA and G6P-derived AGE-BSA. The results are shown below in FIG. 1.

Significantly higher reactivity was observed for the immunogen AGE-RNAse than RNAse. The anti AGE-RNAse antiserum also reacted with albumin modified by incubation with either glucose or G6P, but not with unmodified albumin, indicating the presence of antibodies specific for AGEs.

EXAMPLE 1

Elisa Assays

To monitor the formation of anti-AGE antibodies, rabbit antiserum produced as described above, was titered in a non-competitive ELISA system. RNAse, AGE-RNAse, and AGE-BSA were used as the absorbed antigens.

The absorbed antigen was contacted with rabbit serum, allowing the anti-AGE antibodies contained in the serum to complex. The level of complex formed was then evaluated by adding anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Organon Technica, Durham, N.C.). The titre for anti-(AGE-RNAse) antiserum was thereafter defined as the serum dilution giving a 50% of maximum OD_{405} signal.

Ligand inhibition and AGE measurements were then performed in competitive ELISA. Ninety-six well microtitre plates (Nunc Immunoplate, Gibco, Grand Island, N.Y.) were coated with AGE-BSA (obtained as described above) by adding 0.1 ml of a solution of AGE-BSA (10 μg/ml, dissolved in PBS) to each well and incubating for 2 hrs. at room temperture. Wells were then washed three times with 0.15 ml of a solution containing PBS, 0.05% Tween-20, and 1 mM NaN_3 (PBS-Tween).

The wells were blocked by incubation for 1 hour with 0.1 ml of a solution of PBS containing 2% goat serum, 0.1%

BSA, and 1 mM NaN_3. After washing the blocked wells with PBS-Tween, 50 μl of a competing antigen was added, followed by 50 μl of the rabbit-derived antiserum (final dilution, 1/1000).

Plates were thereafter incubated for 3 hours at room temperature, after which the wells were washed with PBS-Tween and developed with an alkaline phosphatase linked anti-rabbit IgG (raised in goats) utilizing p-nitrophenylphosphate as the colorimetric substrate.

Results were expressed as B/Bo, wherein Bo is the maximum amount of antibody bound in the absence of competing antigen, and B is the amount of antibody bound in the presence of competing antigen. Both Bo and B have been adjusted for background (See Robard, *CLIN. CHEM.*, 20: 1255–1270 (1974) and calculated as [experimental optical density at 405 nm—background optical density (no antibody)]/[total (no competitor)—background optical density].

It was determined that three micrograms of the glucose-derived AGE-BSA standard inhibited antiserum binding by 50%. This standard yielded an A_{350} of 12 mM^{-1} albumin.

The specificity of the anti-(AGE-RNAse) antiserum was thus tested using a variety of AGE-modified proteins, unmodified proteins, and synthetic AGEs. As shown in FIG. 2 (A) results were obtained by testing anti-(AGE-RNAse) antiserum against different modified albumins in a competitive ELISA system using AGE-BSA as the absorbed antigen. All points represent the mean of triplicate determinations.

The AGE-albumin prepared by incubation of BSA with glucose showed the greatest inhibition, followed by AGE-albumin prepared with glucose-6-phosphate and with fructose.

FFI-BSA, a BSA derivative which carries the synthetic AGE ligand FFI, was not recognized by the antiserum. Other albumin modifications such as formylation or maleylation, which produce specific recognition signals for albumin uptake in vivo likewise did not demonstrate any cross-reactivity with the antiserum.

The AGE-modification competed for antibody binding in the competitive ELISA when it was present on diverse carrier proteins (FIG. 2, B). Thus, G6P-derived AGE-HSA, glucose-derived AGE-LDL, and glucose-derived AGE-collagen (type IV), all demonstrated specific inhibition of antibody binding to glucose-derived AGE-BSA. In contrast, unmodified HSA, unmodified LDL, and unmodified collagen did not so compete. Acetyl-LDL, a modified form of LDL which is specifically recognized and taken up by cellular scavenger receptors, also was not recognized by the anti-(AGE-RNAse).

The anti-AGE antiserum was also tested for competition against model, structurally defined AGEs (FIG. 2, C). The model AGE products tested were FFI, AFGP, pyrraline, carboxymethyllysine, and pentosidine. These compounds were isolated from in vitro incubations of amines with reducing sugars or from tissue collagen after reduction and acid hydrolysis. These compounds were tested at high concentrations so that even low levels of inhibition would be readily detected. None of these products competed with the binding of the antiserum to AGE-BSA.

Glucose and G6P were incubated with lysine to determine whether low molecular weight AGEs could react with the antiserum obtained. These products were generated by the incubation of glucose or G6P with lysine in vitro without chemical reduction or hydrolysis. The resulting product was tested for reactivity in the competitive ELISA.

Control incubations consisting of either sugar or lysine alone failed to show any competition (data not shown). Two model Amadori products, deoxypropylaminofructose and deoxymorpholinofructose were also tested, and each compound failed to inhibit antiserum binding. Further evidence that Amadori products are not recognized by the antiserum was provided by the fact that sodium borohydride reduction of AGE-BSA did not diminish reactivity in the competition ELISA (data not shown).

To further characterize the nature of the in vivo-generated AGEs which react with the anti-(AGE-RNAse) antiserum, AGEs were synthesized in the presence of the advanced glycosylation inhibitor aminoguanidine. Aminoguanidine is a hydrazine-like compound which reacts at an intermediate stage of the advanced glycosylation process, inhibiting the formation of protein-bound fluorescent products and crosslinks. As shown in FIG. 4, the inclusion of aminoguanidine in an in vitro incubation of glucose and BSA significantly inhibited the formation of AGEs which react and bind to the anti-AGE antiserum.

EXAMPLE 2

Age Formation Kinetics

The kinetic relationship between the formation of AGE-associated fluorescence and the formation of products which bind to anti-AGE antiserum was also evaluated. FIG. 3 shows a time course for the development of fluorescence and anti-AGE antibody reactive material.

BSA was incubated with glucose, aliquots were removed at various intervals, dialyzed to remove unbound products, and then assayed. AGE-fluorophores were observed to form rapidly between 0 and 40 days and to precede the formation of antibody reactive products.

When measured by ELISA, AGEs were not detected until day 20, and then formed rapidly between days 30 and 70. The formation of both AGE fluorophores and antibody reactive products eventually plateaued.

EXAMPLE 3

Diabetes Evaluation

As stated above, the present invention affords a particularly effective means for the detection and evaluation of diabetes as well as other disease states in which AGE levels are abnormal. Effective assessment of the presence and/or quantity of AGEs in diabetic tissue, and the use of the AGE assays described herein to characterize the overall condition of a mammal known to be diabetic, are described below.

To determine whether tissue AGEs could be measured by anti-AGE ELISA, rats with experimentally-induced diabetes mellitus were evaluated.

Diabetes was induced in 8-week-old male Lewis rats by the rapid intravenous injection of alloxan (40 mg/kg) or streptozotocin (65 mg/kg). Hyperglycemia was confirmed by assaying blood glucose. Blood glucose was determined at 16 week intervals and averaged 20.5±2.4 mM in the alloxan-treated animals (n=24) and 23.5±3.9 in the streptozotocin-treated animals (n=24). There was no significant change in blood sugar levels with time in the control, alloxan-treated, or streptozotocin-treated animals.

At 4 month intervals, 6 animals were sacrificed and the aortas removed and frozen at −80° C. for later analysis. Arterial tissue was slowly thawed, rinsed with PBS, and finely minced with scissors. Lipids were extracted with acetone/chloroform (1:1) by shaking gently overnight at 4° C. Samples then were dried by vacuum centrifugation and

resuspended in 0.2M $NaPO_4$ buffer (pH 7.4). Collagenase (Type VII) was then added at a 1/100 (w/w) ratio and the mixture incubated for 48 hr at 37° C. with mild shaking. One drop of toluene was included to maintain sterility. Digested samples then were centrifuged at 15,000×g and the clear supernatants used for fluorescence, AGE, and hydroxyproline measurements.

In the diabetic animals (alloxan-plus-streptozotocin group), relative fluorescence increased from 13.7%±2.4% at 16 weeks to 23.7%±3.0% at 64 weeks (P<0.001). Fluorescence in the control, non-diabetic animals increased slightly during this time period (8.3%±1.0% to 9%±0.8%, not statistically significant). Tissue and serum AGE values were expressed as AGE Units. One AGE Unit was defined as the amount of antibody-reactive material that was equivalent to that in 1 μg of the AGE-BSA standard. The P values were calculated by the unpaired Student's t-test for comparison between groups.

Analysis of the AGE content by ELISA showed an approximately two-fold increase with time in the diabetic animals (4.8±0.5 U/mg at 16 weeks versus 10.5±1.8 U/mg at 64 weeks, P<0.001). Arterial AGE content also increased with time in the control, non-diabetic rats although at a much lower rate than in the diabetic animals (2.5±0.6 U/mg at 16 weeks versus 4.3±0.4 U/mg at 64 weeks P<0.001).

Human serum was also obtained from normal and diabetic patients. Patients with compromised renal function were also studied because this group of patients has been found to have markedly elevated levels of circulating, serum AGEs.

These circulating, serum AGEs are primarily in the form of low-molecular peptides which are inefficiently cleared by hemodialysis therapy.

Serum samples were obtained from non-diabetic (n=12), diabetic (n=21), and diabetic patients on hemodialysis (n=16). Serum was diluted three-fold with PBS and filter sterilized through a 0.22 μm Millipore filter, (Millipore, Bedford, Mass.) prior to analysis.

When expressed as AGE U/ml, the normal patients (non-diabetic, normal renal function) had a mean level of 10.5±1.3 U/ml serum. The AGE levels were elevated more than two-fold in the diabetic patients (24.7±2.4 U/ml, P<0.001 for diabetic (DM) vs. normal (NL) and almost eight-fold in diabetic patients on hemodialysis (79.4±9.9 U/ml, P<0.001 for DM+HD vs. DM). These results correlate well with the findings of a recent study which utilized a radioreceptor assay to measure the AGE-peptide content of serum obtained from diabetic patients and from diabetic patients on hemodialysis.

EXAMPLE 4

An assay of time-integrated blood glucose levels was performed using the protocol followed in Example 3 above, with samples taken from normal and diabetic patients. The results of the assay were compared against values that are received when time-integrated blood glucose is measured using the known standard of HbA_{1c}, and are presented in FIGS. 7 and 8.

Referring to FIGS. 7 and 8, it can be seen that the performance of the present assay with AGE-hemoglobin as the standard compares favorably with the known determinant and standard HbA_{1c}, and can be used in any instance where the latter test may be called for. The data measurements that were received are virtually identical and the clinical integrity of the present assay is consequently high.

A further diagnostic application of the present invention is in the measurement of fructose-derived AGEs. The measurement of fructose-derived AGEs is being recognized as a significant determinant of the rate of AGE formation, and the concomitant development and extent of the pathologies and other sequelae that have been associated with this reaction, such as diabetes mellitis. Suarez et al., (1989) *J. BIOL. CHEM.*, 264:3674–3679, and McPherson et al., (1988) *BIOCHEMISTRY*, 27:1901–1907, both suggest that the presence and participation of fructose in protein crosslinking fortells a significant role for fructose-derived AGEs that commends its measurement and control, and significantly, Ahmed et al., (1992) *CLIN. CHEM.*, 38(7):1301–1303, state that fructosylated Hb is incapable of effective diagnosis by presently known clinical assays.

Accordingly, the present invention is appropriately extended to the measurement of fructose-derived AGEs in a comprehensive effort to better understand and treat the adverse effects of the reaction of the accumulation of fructose-derived AGEs with body proteins.

EXAMPLE 5

Anti-AGE antibodies developed for the detection of in vivo-formed AGEs were used in a competitive ELISA to measure hemoglobin-linked AGEs in red cell hemolysates.

FIG. 9 shows the results of this analysis for 32 red cell samples obtained from diabetic individuals (DM) and non-diabetic normoglycemic individuals (NL). Hemoglobin-linked AGEs were detected in both groups of individuals, but significantly higher amounts were present in the diabetic group (NL [n=9]: 4.3±0.3 Units AGE/mg Hb; DM [n=23]: 7.7±0.6 Units AGE/mg Hb, [Mean±S.E.], P<0.001 by Student's unpaired t-test).

Antibody reactivity, expressed in AGE Units, was calculated relative to a synthesized AGE-albumin standard. Additional experiments showed that the hemoglobin-AGE modification is stable to dialysis, acid precipitation and proteolysis, and is unaffected by borohydride reduction (data not shown). These data, together with previous studies of anti-AGE antibody specificity confirm that the hemoglobin-AGE moiety is a stable glucose-derived post-Amadori product. The levels of red cell HbA_{1c} correlate with the levels of Hb-AGE in a statistically significant manner.

EXAMPLE 6

The formation of Hb-AGE from hemoglobin and glucose was confirmed in vitro. Purified human hemoglobin was incubated at 37° C. with glucose concentrations that mimicked normoglycemia (5 mM) and hyperglycemia (20 mM). Hemoglobin-AGE formed in a time and concentration dependent manner. See FIG. 11. That early Amadori glycation products are unreactive with anti-AGE antibodies was confirmed in the present system by the observation that sodium borohydride reduction which alters the Amadori product epitope did not affect the detection of Hb-AGE products once formed. The addition of aminoguanidine prevented the formation of hemoglobin-associated AGEs.

Hemoglobin-AGE measurements were performed in blood specimens obtained from patients undergoing treatment with aminoguanidine. The patient group consisted of 18 individuals with long standing diabetes mellitus. Blood samples were obtained before and after 28 days of aminoguanidine treatment and the Hb-AGE levels were determined by ELISA. As shown in FIG. 12, the mean Hb-AGE value decreased significantly as a result of aminoguanidine therapy. (13.8±0.8 Units AGE/mg Hb vs. 10.0±0.9 Units AGE/mg Hb [Mean±S.E.] P<0.001, by Student's paired t-test).

HbA_{1c} values were not affected by aminoguanidine treatment (10.1%±0.8% v. 9.2%±0.8%, [Mean±S.E.] P=NS). No significant changes in either the Hb-AGE or HbA_{1c} levels were observed in blood samples obtained from a group of six patients receiving a placebo control (data not shown).

The existence of an AGE-modified hemoglobin is noteworthy in several respects. First, AGEs generally have been considered to require a time course of months to years to form, even under hyperglycemic conditions. The present findings indicate that within the lifespan of circulating red cells, e.g. about 120 days, significant amounts of AGE-modified hemoglobin are formed. If Hb-AGE Units, expressed relative to a synthetic AGE-albumin standard, are recalculated as a fraction of total red cell hemoglobin, Hb-AGE appears to account for 0.42±0.07% of circulating hemoglobin. This level increases to a mean of 0.75±0.08% in the diabetic group that was studied. These values contrast with corresponding HbA_{1c} fractions of 5.8% and 8.9% for the normoglycemic and diabetic groups respectively.

The high amount of AGE accumulation on hemoglobin compared to connective tissue or basement membrane collagen may reflect the receptor-mediated turnover of connective tissue AGEs during normal remodeling or indicate an inherently enhanced rate of AGE formation on hemoglobin as a protein substrate. Alternatively, circulating red blood cell hemoglobin may be susceptible to modification by reactive plasma AGEs which occur in elevated amounts in patients with diabetes, renal insufficiency or other disease conditions. Irrespective of the mechanism of formation, the application of quantitative ELISA methods to measurements of in vivo-generated AGEs indicates that AGE formation occurs more rapidly with hemoglobin than with connective tissue collagen.

Hb-AGE may thus serve as a useful biochemical index of advanced glycosylation in vivo. The formation of Hb-AGE reflects a time integral of blood glucose concentration that is significantly longer than that established for HbA_{1c} (3–4 weeks). Four week pharmacological intervention with aminoguanidine was sufficient to lower significantly (28%) the Hb-AGE levels in a treated diabetic population.

Hb-AGE measurements may facilitate a variety of investigations into the pathophysiology of both diabetes and age-related complications. These would include clinical studies aimed at elucidating the benefit of strict glucose control in preventing diabetic complications, as well as experimental investigations of the role of advanced glycosylation in the pathogenesis of such diabetes- and age-related conditions as atherosclerosis, hypertension and renal disease.

EXAMPLE 7

Effect of Aminoguanidine on Urine AGE Levels in Normal and Diabetic Rats

Groups of normal and streptozotocin-diabetic rats were left untreated for 11 weeks. Half of the animals in each of the two groups were then started on daily treatment with 70 mg/kg aminoguanidine hydrochloride (AG HCl), by gavage, and the other half of the animals with distilled water by gavage. After 10 additional weeks, urines were collected from all animals over a 24 hour period.

Urine samples were centrifuged and the supernatants were diluted eight-fold with 0.3M potassium phosphate buffer, pH 7.4. Samples of the diluted urines were run in the AGE ELISA assay described above. The results of these measurements are set forth in the Table, below.

TABLE

Treatment	Urine AGEs: Units excreted per 24 hrs.
Normal rats	5400 ± 1200
Normal rats treated with 70 mg/kg/day AG HCl	2200 ± 190
Diabetic rats	9400 ± 340
Diabetic rats treated with 70 mg/kg/day AG HCl	4100 ± 1200

From the above, it was observed that twenty-one weeks of diabetes produced a 1.7-fold increase in urinary AGE excretion over that of normal animals which was normalized by aminoguanidine administration. Aminoguanidine-treated normal rats showed a 60% inhibition of urinary AGE excretion. The above data accordingly confirms the relevance and value of the measurement of urinary AGEs and AGE-peptides to monitor conditions such as diabetes where the turnover of tissue AGEs is a clinically valid long-term determinant, as well as the apparent efficacy of aminoguanidine as a therapeutic agent.

This invention may be embodied in other forms or carried out in other ways without departing from the spirit or essential characteristics thereof. The present disclosure is therefore to be considered as in all respects illustrative and not restrictive, the scope of the invention being indicated by the appended claims, and all changes which come within the meaning and range of equivalency are intended to be embraced therein.

What is claimed is:

1. A pharmaceutical composition containing an anti-AGE antibody in combination with a pharmaceutically acceptable carrier;

wherein said anti-AGE antibody is reactive with in vivo-produced advanced glycosylation endproducts and has the following characteristics:

i. it reacts with an immunological epitope common to said in vivo-formed advanced glycosylation endproducts;

ii. it is cross reactive with advanced glycosylation endproducts formed in vitro; and

iii. it is not cross reactive with the following model advanced glycosylation endproducts however formed: 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrrole (AFGP), 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde (pyrraline), carboxymethyllysine, and pentosidine, wherein reactivity is detected in a competitive immunosorbent assay format, wherein bovine serum albumin (BSA)-AGE obtained by incubation of BSA with glucose is adsorbed to said solid phase, and said model AGE is tested as the inhibitor of binding of said antibody to said BSA-AGE.

2. The pharmaceutical composition of claim 1 wherein said in vivo-produced advanced glycosylation endproducts are selected from the group consisting of Hb-AGE, AGE-HSA, serum AGE-peptides, urinary AGE-peptides, and combinations thereof.

3. An antibody reactive with in vivo-formed advanced glycosylation endproducts and having the following characteristics:

A. it reacts with an immunological epitope common to said in vivo-formed advanced glycosylation endproducts;

B. it is cross reactive with advanced glycosylation endproducts formed in vitro; and

C. it is not cross reactive with the following model advanced glycosylation endproducts however formed: 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrrole (AFGP), 5-hydroxymethyl-1-alkylpyrrole-2-carbaldehyde (pyrraline), carboxymethyllysine, and pentosidine, wherein reactivity is detected in a competitive immunosorbent assay format, wherein bovine serum albumin (BSA)-AGE obtained by incubation of BSA with glucose is adsorbed to said solid phase, and said model AGE is tested as the inhibitor of binding of said antibody to said BSA-AGE.

4. The antibody of claim 3 wherein the advanced glycosylation endproducts formed in vitro and reactive with said antiserum are selected from the group consisting of AGE-RNAse, AGE-hemoglobin, AGE-albumin, AGE-LDL, AGE-collagen Type IV, AGE-BSA reduced with $NaBH_4$, and mixtures.

5. The antibody of claim 3 prepared by hyperimmunizing a mammal with a foreign protein or the reaction product of a protein incubated with a reducing sugar.

6. The antibody of claim 5 wherein said reaction product of a protein incubated with a reducing sugar is AGE-RNAse.

7. The antibody of claim 3 wherein said epitope is formed by the incubation of a reducing sugar with a proteinaceous material selected from the group consisting of RNAse, hemoglobin, lysine, collagen Type IV, LDL, BSA and HSA.

8. The antibody of claim 7 wherein the reducing sugar is selected from the group consisting of glucose, glucose-6-phosphate, fructose, and ribose.

9. The antibody of claim 3 wherein said epitope is formed by the incubation of glucose with RNAse.

10. The antibody of claim 3 which is polyclonal.

11. The antibody of claim 3 which is monoclonal.

12. A labeled antibody reactive with in vivo-formed advanced glycosylation endproducts and having the following characteristics:

A. it reacts with an immunological epitope common to said in vivo-formed advanced glycosylation endproducts;

B. it is cross reactive with advanced glycosylation endproducts formed in vitro; and

C. it is not cross reactive with the following model advanced glyeosylation endproducts however formed: 2-(2-furoyl)-4(5)-(2-furanyl)-1H-imidazole (FFI), 1-alkyl-2-formyl-3,4-diglycosyl pyrrole (AFGP), 5-hydroxymethyl-1-alkylpyrrole,-2-carbaldehyde (pyrraline), carboxymethyllysine, and pentosidine, wherein reactivity is detected in a competitive immunosorbent assay format, wherein bovine serum albumin (BSA)-AGE obtained by incubation of BSA with glucose is adsorbed to said solid phase, and said model AGE is tested as the inhibitor of binding of said antibody to said BSA-AGE.

13. The labeled antibody of claim 12 wherein said label is selected from the group consisting of an enzyme, a chemical which fluoresces, and a radioactive element.

14. The labeled antibody of claim 12 which is polyclonal.

15. The labeled antibody of claim 13 which is monoclonal.

* * * * *

Exhibit 7

Applicant C

US005626843A

United States Patent [19]

Skurkovich et al.

[11] **Patent Number: 5,626,843**

[45] **Date of Patent: May 6, 1997**

[54] **TREATMENT OF AUTOIMMUNE DISEASES, INCLUDING AIDS, BY REMOVEL OF INTERFERONS, TNFS AND RECEPTORS THEREFOR**

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[51] Int. Cl.[6] **A61K 39/395**

[52] U.S. Cl. **424/140.1**; 604/6

[58] Field of Search 424/140.1; 436/547; 604/5, 6

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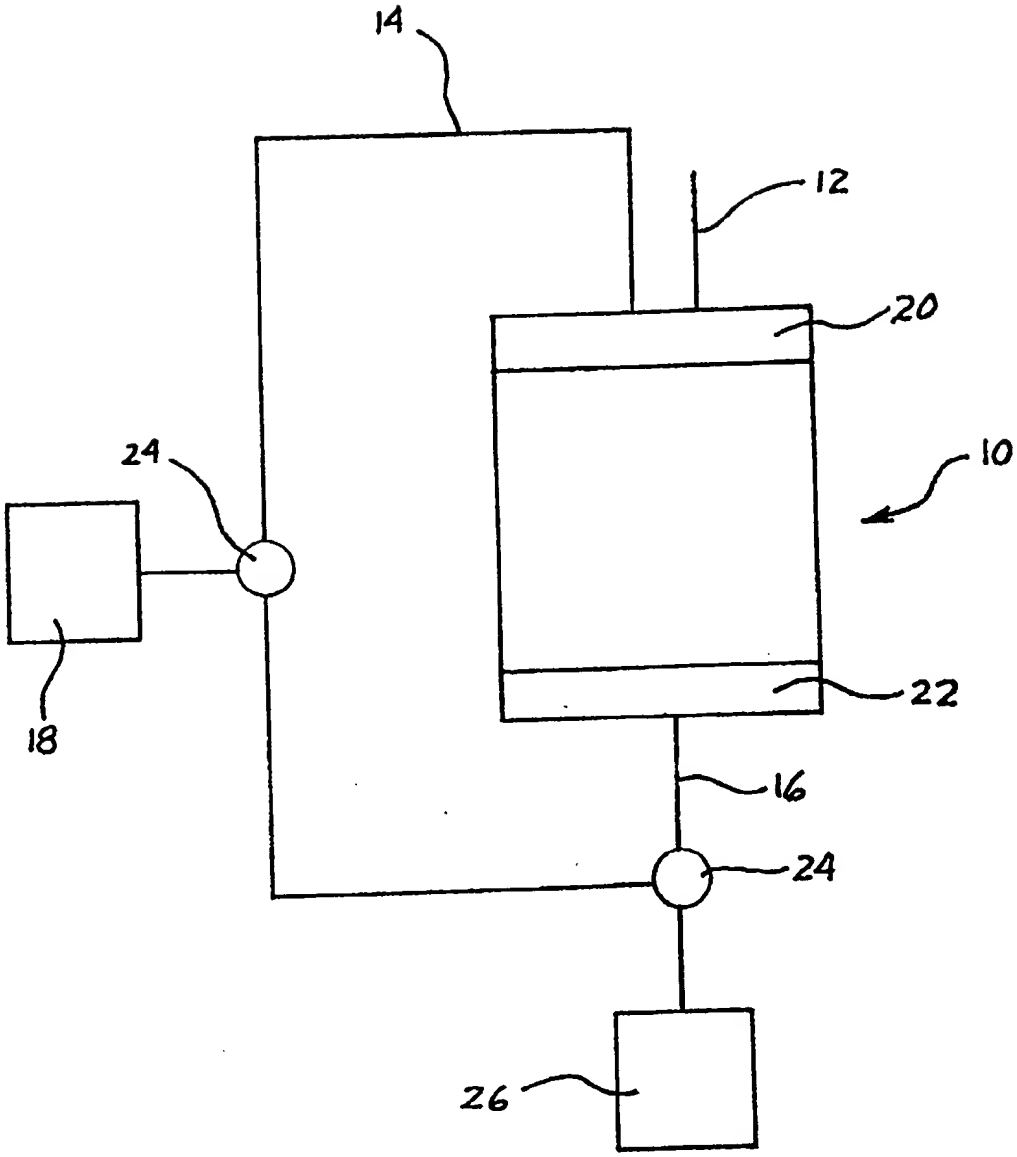
Attorney, Agent, or Firm—Evelyn H. McConathy

[57] **ABSTRACT**

The present disclosure concerns a treatment for autoimmune diseases, including AIDS, by removing interferons, TNFs and receptors therefor, from body fluids. An extracorporeal device exposes fluids from a patient, including blood, plasma, cerelorospinal fluid, and the like, to an immunosorbent to accomplish removal. Following treatment, the fluid is returned to its source.

16 Claims, 1 Drawing Sheet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TREATMENT OF AUTOIMMUNE DISEASES, INCLUDING AIDS, BY REMOVEL OF INTERFERONS, TNFS AND RECEPTORS THEREFOR

BACKGROUND OF THE INVENTION

a) Field of the Invention

The invention concerns a method to treat conditions and diseases which are caused by the disturbance of synthesis of interferons (IFNs) and certain other substances (e.g., tumor necrosis factor) which damage the immune system and have a direct pathological action on cells.

b) Description of Related Art

Besides its anti-virus and anti-proliferative roles, IFN also plays a role as an immunoregulator (Immunology 25: 367, 1973; W. Stewart, Interferon System. 1979). The normal functioning of the interferon system in vivo is critical for the normal functioning of the immune system. A change in IFN synthesis can bring about a change in the immune system. In 1974 an article was published suggesting that the hyperproduction of IFN can be the main cause of the development of autoimmune disease (Nature 551: 2047, Feb. 22. 1974). IFN was found in the circulation of patients with autoimmune diseases and it was neutralized in vivo with antibody to leukocyte (alpha) IFN; healthy people do not have interferon in their blood (Annals of Allergy 35: 356. 1975).

Later it was shown that this hyperproduced alpha IFN is found not only in the circulation of patients with classic autoimmune diseases, but also in patients with HIV infection (J Infec Dis 146: 451, 1982). This alpha IFN is pH labile while normal alpha IFN is pH stable. This aberrant type of IFN may participate in the pathogenesis of AIDS where its presence is a predictive marker of AIDS progression (Cancer Res 46: 417, 1986). It was proposed to remove aberrant IFN from the circulation as a method of treatment of patients with autoimmune disease and AIDS, also considered an autoimmune disease.

Aberrant IFN can induce tumor necrosis factor (TNF) and its receptors (AIDS Research and Human Retroviruses 7: 545. 1991), which enhances virus replication (Proc Natl Acad Sci USA 86: 2365, 1989). In other words, aberrant IFN production reflects virus replication. The aberrant IFN induced by HIV has low anti-(HIV) viral activity (J Immunol 148: 422, 1992). In some stages of AIDS, its induction may help the virus survive.

Alpha IFN, mostly the aberrant type, may be the main cause of the development of autoimmune disease, but in some situations gamma IFN, and more rarely beta IFN, can also play a pathogenetic role since all of these participate in immune regulation. Every hyperproduction and chronic circulation of IFNs, especially aberrant IFNs, and other defective IFNs, have a negative influence on the function of different body systems, in particular the immune system (J of Clin Immunol and Immunopathol 43: 362, 1987; Am J of Med Sci 295: 532, 1988). The presence in AIDS patients of gamma IFN may be a marker of the intensification of the autoimmune process.

Besides being present in the circulation, IFNs have also been found in the cerebrospinal fluid in some patients with psychiatric and neurologic diseases (Acta Biol Med Germ 38: 879, 1979; Acta Neurol Scand 53: 152, 1976) and in the joint synovial fluid (Ann of Rheum Dis 42: 672, 1983) of patients with rheumatoid arthritis. Healthy people do not have interferons in the spinal or synovial fluids.

As is known, AIDS is induced by HIV. In autoimmune disease, where HIV like particles and their antibodies against them were found in the sera of humans and in animal models, it is possible that the particles are integral parts of the lymphocytes or organs. HIV and its particles are interferon inducers. Every antigen is an interferonogen; "self" cannot induce IFN. Thus, the production of IFN signals the invasion by a foreign antigen. IFN production, mostly IFN of the aberrant type, and its prolonged circulation in the body is an inseparable part of the development of autoimmune disease and triggers immunological chaos. For example, antibodies to CD4 in patients with HIV infection (Am J of Med 78: 621, 1985), can crossreact with class II antigen (Proc Natl Acad Sci USA 88: 3060, 1991) which in turn is induced by gamma IFN, or by gamma IFN in combination with TNF, and possibly by aberrant IFN, which induces TNF. In other words, aberrant IFN and TNF could be important pathological triggers of immune dysregulation in AIDS.

Besides classic AD and AIDS, there are a number of other pathological conditions in which autoantibodies play a pathogenic role. After cell (or organ) transplantation and after heart attack or stroke, certain antigens from the transplantation of cells (organs) or necrotic cells from the heart or the brain can stimulate the production of antibodies or immune lymphocytes (R. Johnson, L. Lynne and Wm. Seldin, Sem Nuc Med 19: 238, 1989; M. Leinonen, E. Linnanmaki, K. Mattile and M. S. Nieminen, et al., Microbiolo Path 9: 67. 1990; J. Montalban, A. Codin, J. Ordi, M. Vilardell, et al., Stroke 22: 750, 1991), which later participate in rejection (in the case of a transplant) or attack the cardiac and brain cells, aggravating the condition. Every antigen stimulation is accompanied by the immediate synthesis of interferon which triggers the immune process. To counter transplant rejection, antibodies to three kinds of interferons (alpha, beta, and gamma), or in some cases, gamma IFN alone, and the antigen of the transplanted cell or organ are placed in the immunosorbent column. To treat infarction or stroke, antibodies to IFNs as well as cardiac or brain antigens are placed in the immunosorbent column. Further, the present invention may be used in combination with immunosuppressive therapy necessary for treating infarction and stroke.

In addition, in human autoimmune disease certain cells express abnormally elevated levels of HLA class II antigens, which is stimulated by the disturbed production of gamma IFN and TNF (IFN 9, Academic Press 1987, p. 75). The disturbance of the synthesis of HLA class II antigen plays an important role in the pathogenesis of autoimmune disease and AIDS. The disturbance of HLA class II antigen leads to a disturbance of the presentation of antigens to T cells, T/B cooperation and the dysregulation of the interaction between T cells. For the normalization of the immune system, it is necessary to remove hyperproduced class II antigens, and in some cases, it is also necessary to remove its receptors. For this reason, the present invention includes in the immunosorbent antibody to class II antigens, and in some cases, antibody to its receptors. This absorption can be obtained from whole blood or from the plasma with leukocytes

SUMMARY OF THE INVENTION

An objective of the present invention is to restore immunity in some autoimmune diseases, such as AIDS, by removing IFNs together with TNF, and in some cases the receptors therefor, as well as certain antibodies and antigens, using combinations of immunosorbents in an extracorporeal device. An example of antibody use is placing antibody to HLA class II antigen in the immunosorbent. An example of using antigen in an immunosorbent is the antigen—CD4.

The latter can be included among the immunosorbents, either in combination with other sorbents, or alone. For example, in treating AIDS patients, a combined sorbent comprising a first component of antibodies to IFNs, a second component of lymphocytes from healthy donors, mostly CD4 cells to absorb serum antibodies which react with CD4 cells, and a third component to remove TNF can be used. In rheumatoid arthritis, antigens from joints, skin and possibly other target antigens can be used as sorbents in addition to antibodies to IFNs and other sorbents. In autoimmune diseases of the central nervous system, additional immunosorbents antigens from the brain cells could be used to absorb antibodies formed against brain cells. In treating patients with systemic lupus erythematosus, in addition to other sorbents, a sorbent (DNA) can be added to absorb anti-DNA antibodies.

In still another example of treating an autoimmune disease, e.g., rheumatoid fever, the invention uses one sorbent for removing IFNs and other substances, often together with their receptors, and a second sorbent for removing antibodies against cardiac tissue. The second sorbent can also include both certain serotypes of streptococcus (streptococcus group "A") and antigens of cardiac tissue. Some antigens from cardiac tissue and some serotypes of streptococcus are antigenically similar. When treating certain diseases connected with hypersensitivity of the immediate type, e.g., bronchial asthma, a combined sorbent having a first component for absorbing IFNs and other substances, as well as a second component made of an antibody against IgE (immunoglobulin E), can be used.

These approaches could restore the immune system and, especially in AIDS, break the chain of events which are insuring the continuous replication and survival of the virus, HIV.

Removal or neutralization of alpha, gamma and beta IFNs, TNF, and HLA II class antigen, and/or their receptors in some cases, as well as other components, can be accomplished with polyclonal or monoclonal antibodies produced in mice or human hybridoma. These different antibodies can also be put in an immunosorbent column. If the antibody is to be administered intramuscularly or intravenously, then it is preferable to use monoclonal antibody produced in human hybridoma (any monoclonal antibody, however it is produced, as long as it comprises human protein). To neutralize antibodies to different cell antigens, the antigens from these cells must be placed directly in the immunosorbent column. It is also possible to use a combined treatment—with both the immunosorbent column and parenteral injection of specific antibody.

Regarding the extracorporeal system, these substances can be obtained with an extracorporeal device from whole blood, plasma with leukocytes, or plasma only. To do this, one can use a blood cell separator (e.g., Cobe "Spectra") to which the immunosorbent column is connected. Such a device is known from U.S. Pat. No. 4,362,155 which is incorporated herein by reference. To remove pathological substances from joint and spinal fluids, a special extracorporeal device with a small amount of immunosorbent is used.

BRIEF DESCRIPTION OF THE DRAWING

The sole figure shows an extracorporeal device for removing pathological substances from joint and spinal fluids.

DESCRIPTION OF THE PREFERRED EMBODIMENT

The column **10** and tubing are made of plastic approved for the use of blood. The total volume of the column **10** and tubing is small, approximately 30–35 ml. The column **10** is filled with the immunosorbent, consisting of the antigen or antibody bound to Sepharose 4B or another suitable matrix, through a short filling tube **12** placed at a first end of the column **10**. Input tube **14** and return tube **16** are connected between the first end and a second end, respectively, of the column **10**, and a fluid sample **18**. A first filter **20** is interposed between the input tube **14** and the column **10**, and a second filter **22** is interposed between the return tube **16** and the column **10**. The filters **20**, **22** prevent the flow of immunosorbent from the column **10**. Two way stopcocks **24**, **24** regulate flow throughout the system.

The column **10** is positioned lower than the source of the fluid sample **18**, whereupon the cerebrospinal or synovial fluid flows into the column **10** under the influence of gravity. After the fluid perfuses through the immunosorbent, it is collected in a holding tube **26** from which it is returned to the source of the fluid.

Materials according to the present invention to be used with the column **10** may be obtained as follows:

EXAMPLE 1

Production of antibody to human gamma IFN: Adult rabbits are immunized with purified human gamma IFN (10^5–10^6 unit/mg protein). This interferon is first mixed with equal volumes of Freund's Complete Adjuvant and 30% Arlacel A and injected IM or subcutaneously on day 1, 4, 14 and 43 (100 units, 200 units, 200, 200 respectively). After this 200,000 units/month for 6 additional months are injected. The serum is obtained when the titer has reached 100 units (1 unit of antibody neutralizes 10 units of gamma IFN). IgG is isolated and purified.

EXAMPLE 2

Production of antibody to human beta IFN: Adult sheep receive 12 weekly injections of 10^7 of human beta IFN. Six weeks after the 12 injections a booster shot of 2.7×10^7 units of IFN is given which is first mixed with Freund's Complete Adjuvant and injected into several IM sites. Bleeding begins 7 days later. IgG is then obtained and purified.

EXAMPLE 3

Column Preparation: Sepharose CL-4B (Pharmacia, Piscattawy, N.J.) (100 ml) is washed thoroughly with pyrogen free water, then suspended in 300 ml ice cold 1M $NaCO_3$ pH 11.0. 20 gms CNBr in 10 ml acetonitrile is added to the Sepharose. After 2 minutes this is collected on a fretted glass funnel. Sepharose cake is washed with 5 volumes of ice cold 0.2M Na Bicarbonate buffer pH 9.5 and 5 volumes of ice cold 0.5M Na Bicarbonate buffer pH 8.5. This is immediately resuspended in a solution of 780 mg anti-alpha IFN antibody in 200 ml of 0.2M Bicarbonate buffer pH 9.3. This is incubated for 20 hours at 4 degrees C. This is then centrifuged, the supernatant is decanted and sediment is resuspended in 100 ml of 0.05 PBS (Phosphate buffered saline) and 2M glycine pH 8.0 for 12 hours at room temperature. This is then washed thoroughly with 20 volumes of PBS.

What is claimed is:

1. A method of removing antigens from a patient with autoimmune disease or AIDS comprising the steps of:

drawing fluid from said patient;

passing said fluid through immunosorbent comprising a combination of antibodies, consisting essentially of: (a) one or more antibodies to at least one interferon, selected from the group consisting of alpha interferons and gamma interferons, and receptors therefor; and (b) one or more antibodies to tumor necrosis factors, and receptors therefor; and

returning said fluid to said patient.

2. The method according to claim 1, wherein said method removes alpha interferon and tumor necrosis factor from said fluid.

3. The method according to claim 1, wherein said method removes gamma interferon and tumor necrosis factor from said fluid.

4. The method according to claim 1, wherein said method removes both alpha and gamma interferon, and tumor necrosis factor, from said fluid.

5. The method according to claim 1, wherein said fluid is selected from the group consisting essentially of blood, plasma, plasma containing leukocytes, peritoneal fluid, cerebrospinal fluid, and synovial fluid.

6. The method according to claim 1, wherein said antibodies are selected from the group consisting of monoclonal antibodies, polyclonal antibodies, and combinations thereof.

7. The method according to claim 1, wherein said method removes: (a) one or more receptors for at least one interferon, selected from the group consisting of alpha interferons and gamma interferons, and (b) tumor necrosis factor, from said fluid.

8. The method according to claim 1, wherein said method removes: (a) one or more interferons, selected from the group consisting of alpha interferons and gamma interferons, and (b) at least one receptor for tumor necrosis factor, from said fluid.

9. The method according to claim 1, wherein said method removes (a) one or more receptors for at least one interferon, selected from the group consisting of alpha interferons and gamma interferons, and (b) at least one receptor for tumor necrosis factor, from said fluid.

10. A method of removing antigens from a patient with autoimmune disease or AIDS comprising the steps of:

drawing fluid from a patient;

passing said fluid through immunosorbent comprising in combination a plurality of antibodies, consisting essentially of at least one antibody selected from the group consisting of anti-alpha interferon and antibody to alpha interferon receptor, and at least one antibody selected from the group consisting of anti-gamma interferon and antibody to gamma interferon receptor; and

returning said fluid to said patient.

11. The method according to claim 10, wherein said fluid is selected from the group consisting essentially of blood, plasma, plasma containing leukocytes, peritoneal fluid, cerebrospinal fluid, and synovial fluid.

12. The method according to claim 10, wherein said antibodies are selected from the group consisting of monoclonal antibodies, polyclonal antibodies, and combinations thereof.

13. The method according to claim 10, wherein said treatment removes both alpha interferon and gamma interferon from said fluid.

14. The method according to claim 10, wherein said treatment removes both alpha interferon receptor and gamma interferon receptor from said fluid.

15. The method according to claim 10, wherein said treatment removes a combination of: (a) alpha interferon, and (b) receptor for gamma interferon, from said fluid.

16. The method according to claim 10, wherein said treatment removes a combination of: (a) at least one receptor for alpha interferon, and (b) gamma interferon, from said fluid.

* * * * *

Exhibit 8

Applicant D

United States Patent [19]

Osther

[11] **Patent Number: 5,008,183**

[45] **Date of Patent: Apr. 16, 1991**

[54] **ASSAY SYSTEM FOR DETECTING ANTIBODY AND A METHOD OF PRODUCING NON-HUMAN IMMUNE ANTIBODY**

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[73] Assignee: **Bio-Research Laboratories, Inc., Dallas, Tex.**

[21] Appl. No.: **192,241**

[22] Filed: **May 10, 1988**

[51] **Int. Cl.**[5] **G01N 33/569**

[52] **U.S. Cl.** **435/5**; 435/7.32; 436/513; 436/532; 436/547; 436/815; 530/387; 530/830

[58] **Field of Search** 435/5, 7; 436/513, 532, 436/547, 815; 530/387, 830

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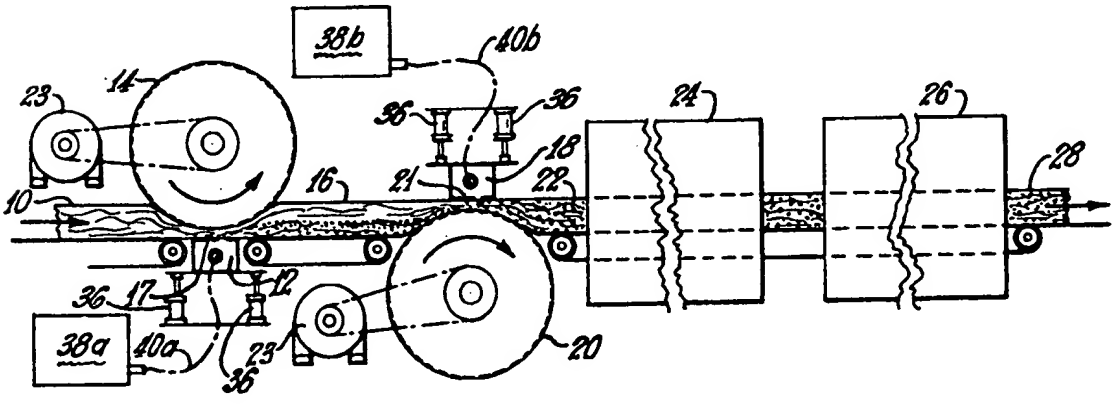
Primary Examiner—Christine Nucker

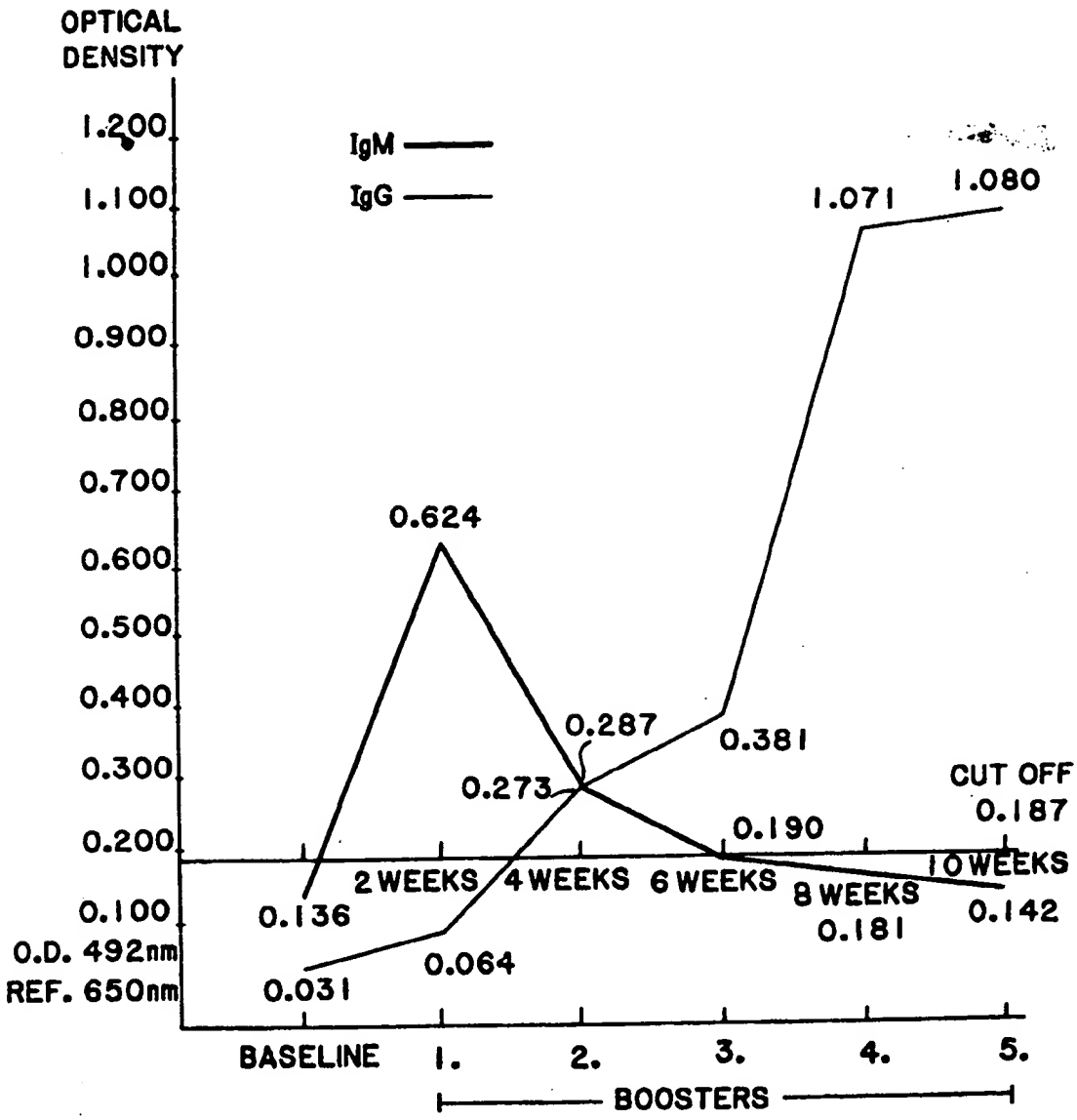
Attorney, Agent, or Firm—Bryan, Cave, McPheeters & McRoberts

[57] **ABSTRACT**

An improved assay method for detecting the presence of an antibody capable of binding with an antigen of a virus is provided. The improvement comprises using a non-human immune antibody which is reactive with an anti-human antibody as a positive control in the assay. Non-human immune IgM and a method of producing the IgM is also provided.

12 Claims, 1 Drawing Sheet

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0.D. 492nm
REF. 650nm
IgM
IgG
0.624
0.136
0.287
0.273
0.381
1.071
1.080
0.190
CUT OFF
0.187
2 WEEKS
4 WEEKS
6 WEEKS
8 WEEKS
10 WEEKS
0.181
0.142
0.064
0.031
BASELINE
1.
2.
3.
4.
5.
BOOSTERS

ASSAY SYSTEM FOR DETECTING ANTIBODY AND A METHOD OF PRODUCING NON-HUMAN IMMUNE ANTIBODY

TECHNICAL FIELD

The present invention relates to the use of a non-human immune antibody, which is capable of reacting with an anti-human antibody, in an assay system for detecting the presence or absence of antibodies to viral and/or bacterial infective agents. In particular, the invention pertains to the use of porcine immune IgG and porcine immune IgM, as a positive control in an assay system. The present invention also pertains to non-human immune IgM, preferably porcine immune IgM and a method of producing it.

BACKGROUND OF THE INVENTION

Assay systems capable of detecting the presence or absence of antibodies generated in response to the presence of antigens are well known. Such assay systems have proved useful in, inter alia, the diagnosis of various diseases. For example, viral infections, such as AIDS (acquired immune deficiency syndrome) and CMV (cytomegalovirus) may be diagnosed with assays which detect the presence of viral antibodies in patients suspected of having the disease. Examples of such assay systems which employ antigen-antibody binding include ELISA, Western Blot, Quick Western Blots (U.S. Pat. Nos. 4,816,387 and 4,885,235) and RIA. Such diagnostics uniformly include controls to insure the integrity of the test system.

Typically, the diagnostics have both positive and negative controls. The positive control provides pertinent information concerning the activitY of the test system, i.e., that reactive antibodies specific to the antigens used in an antibody test system are bound to the antigens (indicating that the antigens used in the test system are working properly), and that the anti-immunoglobulin used to detect the bound immunoglobulin is working. In the case of an ELISA system the anti-immunoglobulin may be labeled with an enzyme (conjugate) which activates a substrate added to the system to give a chromogen reaction; in this case the positive control indicates whether the conjugate has reacted, and whether the substrate has worked properly as an activated chromogen. A negative control provides information about the absence of reactive antibodies specific to the particular antigens used in a test system. It also provides information as to the reaction level, determined by the signal used in a particular test, at which a specimen may be considered negative.

The cut-off point in a particular test is often based upon the relative value obtained by a positive control and/or by the negative control. An acceptable detection range obtained by the controls utilized with a particular type of test kit is specifically designed and titrated for that tYpe of kit. The positive control "value" obtained in a particular test system affects the sensitivity of that test system; the negative control "value" affects the specificity of the test system.

Presently, the antibody used for a positive control is obtained from seropositive human donors. As can be appreciated, it is disadvantageous to depend upon seropositive donors as the sole source of antibody required for a positive control. Notably, the supply of antibodies is scarce and uncertain and the quality and characteristics of the antibody varies from donor to donor. Further, as more successful therapies become known and used, fewer seropositive donors will be available, and thus the required antibody even more difficult to obtain.

In the case of AIDS patients it has been found that the condition of patients who donate blood or are subjected to plasmaphoreisis deteriorates rapidly. Therefore, obtaining AIDS positive blood or plasma from patients can accelerate the disease and accordingly, using AIDS patients as a source of antibody for use as a positive control should be avoided.

The previously mentioned assay systems detect the presence or absence of IgG (immunoglobulin G). Such assays only allow "controlled" detection (measurement defined by use of anti-IgG conjugate and of antibody positive control) of the presence of IgG in blood and body fluids directed to antigens used in the test systems. The appearance of detectable IgG directed to antigens in an infected/immunized individual does not occur until 30–40 days after initial infection in many instances. The IgG class antibodies are often present for months or years after infection/immunization.

The presence of circulating IgG directed to immunizing antigens during the course of an infection (or after immunization) is preceded by the presence of circulating IgM antibodies directed towards the antigens/immunogens. IgM antibodies directed to antigens in an infected/immunized individual are often present in detectable quantities as early as 14 days (or earlier) after the infection/immunization. The IgM class antibodies gradually lose titer 30–35 days after initial infection/immunization.

It is widely recognized that diagnostics which can detect antibodies other than IgG are desirable. For example, it is known that generally after confrontation with a foreign body, the human immune system responds by generating antibodies against the foreign body or antigen. It is believed that IgM, not IgG is produced first. As can be appreciated, assays capable of detecting IgM will facilitate early detection of numerous diseases. IgM is, however, a relatively short-lived antibody. While it may be produced shortly after infection, IgM levels fall, eventually below detectable levels, as IgG is produced, in increasing amounts. See FIG. 1 which compares the titer of IgM and IgG in a pig immunized with HIV-1. Because IgM has a short life span, IgM levels are typically below detectable levels before many diseases are even diagnosed. Therefore, IgM is not readily obtainable from seropositive donors and a dependable, reliable source of this important antibody is needed.

The present invention overcomes the previously mentioned disadvantages because it provides the ability to produce the desired antibody, i.e., IgG and IgM in a non-human species. In accordance with the present invention there is provided a method of using non-human immune antibody as a positive control in assay systems.

SUMMARY OF THE INVENTION

The present invention provides a use for non-human immune antibody in an improved assay system for detecting the presence or absence of antibody which binds with viral antigen. In an assay for detecting the presence or absence of an antibody which binds to an antigen of a human retrovirus comprising the steps of (1) sequentially contacting the antigen with a biological fluid and positive control comprising an antibody to the

antigen for times and under conditions sufficient for the antigen and any antibody in the biological fluid, and the antigen and antibody in the positive control to form antigen-antibody complexes and (2) detecting the formation of the complexes, the improvement comprises the use of a positive control comprising a non-human immune antibody, the non-human immune antibody is reactive with an anti-human antibody.

It is presently preferred to use mammalian immune IgG and/or IgM and most preferably porcine immune IgG and/or IgM as a positive control in the assay.

The present invention also provides non-human immune IgM antibody and a method for producing such IgM.

It has been found, in accordance with the invention, that in both HIV-1 and in HTLV-I lysates immunized animals IgM directed to viral antigens may be detected as early as 12–15 days after immunization. IgM is still detectable 30–40 days after immunization. At that time IgG directed to the viral antigens (30–40 days after immunization) is still measurable. It is anticipated that in humans IgG antibodies directed to HIV-1 do not appear until, at the earliest, about 4 weeks after infection.

DETAILED DESCRIPTION OF THE INVENTION

Producing Immune Antibodies

In accordance with the present invention, the non-human immune antibody is obtained by immunizing an animal with viral material against which it is desired to raise a specific antibody. Examples of suitable immunizing agents include HIV-1, HIV-2, HTLV-I, HTLV-II, CMV and Epstein-Barr Virus. Of course, other immunizing agents as may be known to those skilled in the art are also useful.

The immunization procedure begins with a first vaccination of the animal with a preparation comprising 10–500 ug of viral lysate or selected portions of the viral core or envelope proteins. The viral material is preferably solubilized in Triton X-100, SDS (sodium dodecyl sulfate), mercaptoethanol, and/or Nonidet P40 detergent, and suspended in phosphate buffered saline (PBS), pH 7.2–7.4. It is presently preferred to premix 0.1–5% Triton X-100 with the PBS to elicit an optimum immune response. The first injection contains an adjuvant; Freund's complete adjuvant is preferred for this purpose. Of course, other adjuvants known to those skilled in the art may also be used. The first vaccination typically comprises a total volume of 2 ml, one ml of viral material in buffer plus one ml of adjuvant. The viral material and adjuvant should be thoroughly mixed immediately prior to injection. In a preferred embodiment, the first vaccination contains 50–100 micrograms of viral material in 1.0 ml of PBS-Triton X-100 mixed with 1.0 ml of Freund's complete adjuvant.

Booster immunizations are generally prepared without adjuvant. These injections typically contain 10–500 ug of viral proteins, preferably from about 50–100 ug per injection in PBS, pH 7.2–7.4. The lysate can be solubilized in detergents, e.g., Triton X-100. Booster injections begin from about 7 to 30 days after the initial vaccination and every 7 to 30 days thereafter until the desired antibody titer is measured, i.e., a sufficiently high titer is indicated by an OD value at least equal to a positive control reading on an ELISA test system. For example, in the ElectroNucleonics (VIRGO) HIV-1 Antibody ELISA system an HIV-1 positive control serum has an OD value of over 0.250 and under 1.000 (at 492 nm with a 650 nm reference) measured on a Behring Processor II.

It should be understood that optimal antibody titers may vary depending upon the virus used as an antigen and the particular test system used. The IgG positive control and the IgM positive control should be capable, in a certain dilution, of producing an OD value within the OD ranges given for a positive control in the individual types of known test kits (to be determined for each type of test kit) containing viral antigens for which the positive control is to be used. For example, a nonimmunized control pig serum produces an OD value, which is under the cut-off at the same dilution at which a pig positive control serum gives an acceptable positive value (e.g., for HIV-1 A6 ELISA VIRGO ENI, between 0.25–1.100).

The antibody titer is checked periodically, typically every 14 days or just prior to administering booster injections. A first blood sample is generally drawn and tested prior to the initial vaccination to establish a baseline antibody level.

It is presently preferred to vaccinate by subcutaneous injection, but other modes of administration, such as intramuscular injection may be used. The vaccination site is preferably the side of the neck, typically injecting three to four different locations. Vaccination on the neck is considered to yield higher antibody titers, but other vaccination sites as are known to those skilled in the art are suitable for raising the desired immune antibody.

Once the desired antibody titer is measured, blood from the immunized animal is collected, either by bleeding or sacrificing the animal and collecting its blood. When raising IgM antibody, to insure sufficient high antibody titer for use as a positive control, it is preferred to sacrifice the animal and harvest its blood for separation of serum when the pig serum tested shows an OD value which is within the range of a positive control, when tested in different test systems. However, it is preferable to harvest all the pig blood when the pig serum shows maximum OD value for the particular class of immunoglobulins (IgM or IgG) when the other class of immunoglobulins is below the cut-off point.

The collected blood or serum extracted therefrom is treated before use to inactivate any live virus. It is presently preferred to photochemically treat the blood or serum with Psoralen followed by U.V. irradiation. Other methods such as gamma irradiation or heat inactivation (56° C. for one hour) may also be used. It should be understood, however, that heat inactivation may adversely affect antibody titer and may not be as effective in inactivating virus as the other methods.

It is presently preferred to use serum from the immunized animal as a positive control in an assay system. However, whole blood, plasma or any other form of antibody as may be known to those skilled in the art is also useful. Before use, it is presently preferred to filter and sterilize the blood or serum and then freeze aliquots for later use; alternatively, the serum may be freeze-dried or fractionated to enrich the IgG or IgM fractions.

The previously described method can be used to produce antibody against several viruses including HIV-1, HIV-2, HTLV-I, HTLV-II, cytomegalovirus, Epstein-Barr virus, and hepatitis B virus. It is also considered within the scope of the invention to produce

antibodies against a variety of bacteria such as *E. coli*, Salmonella, Tetanus, Streptococcus, and Neisseria.

The following examples are considered illustrative of the present invention.

EXAMPLE 1

Porcine Immune IgG Against HIV-1

A 60 lb mixed Yorkshire breed pig was immunized (by subcutaneous injections in the left side of the neck) with 50 ug of solubilized HIV-1 antigen lysate, psoralen—U.V. irradiation inactivated, in 1 ml of PBS-Triton X-100 and 1 ml of Freund's complete adjuvant. Just prior to injection blood was drawn from an ear vein and tested for the presence of IgG and IgM antibodies to HIV-1.

Booster injections contained 50 ug of HIV-1 viral lysate in 1 ml of PBS buffer containing lysing detergents such as Triton X-100. The first booster was administered 14 days after the first immunization, and a second booster 14 days later. The pig received a total of 5 vaccinations before it was sacrificed and its blood was harvested, and the serum was used as IgG against HIV-1. Prior to each booster, ear vein blood samples were drawn (and designated Booster #1 and Booster #2, etc.) IgG and IgM antibody titers were measured.

The presence of IgG anti-HIV-1 was measured using the ENI HIV-1 ELISA VIRGO system, following the method described by the manufacturer. The IgM anti-HIV-1 was measured using a modification of the ENI system. The modification comprised substituting affinity purified goat anti-human IgM μ change specific, labelled with alkaline phosphatase (available from Calbiochem, Catalog #401902) for the anti-IgG conjugate normally used. P-Nitrophenyl phosphate tablets, prepared in accordance with the manufacturer's instructions, were used as the substrate (P-Nitrophenyl phosphate tablets are available from Bio-Rad, Catalog #172-1063). The incubation times and washing procedure were performed as directed in the ENI package insert. The results of the IgM class were read on the Behring ELISA Processor II at 405 nm with a 650 nm Reference (see FIG. 1). The test results are plotted on FIG. 1, which is a graph plotting the OD values of IgG and IgM specific anti-HIV-1 against time.

As may be seen in the drawing, IgM is detectable before IgG. IgM peaks at about day 14 and falls to below detectable levels by day 42.

Based on the antibody titer indicated by the OD value at Booster #3, the pig's blood contained an antibody titer sufficient for use as an IgG class positive control 42 days after vaccination. It is presently believed that between two and six booster injections are required to reach antibody titers in the optimal range.

EXAMPLE 2

Producing IgG Specific Anti-HIV-1

A 60 lb mixed Yorkshire breed pig was immunized as described in Example 1 with 50 ug of solubilized HIV-1 antigen lysate. Five booster injections, each containing 50 ug of viral lysate, were administered every fourteen days, beginning 14 days after the first injection. Blood samples were drawn from the pig's ear vein prior to the first vaccination to obtain baseline antibody levels, and prior to administering each booster injection. The pig was sacrificed at day 60 and its blood collected. Serum was separated, sterile filtered and frozen. Upon testing the serum showed a high titer of IgG specific anti-HIV-1 (O.D. about 1.800) with IgM below detectable levels. The serum collected is thus useful as a positive control in an assay for detecting antibodies which bind with HIV-1.

The following test results were obtained:

	Pig IgG Anti HIV-1	Cut-Off	Human HIV-1 Ab Positive Control (ENI)
Baseline*	0.012-0.017	0.273	0.791
Booster #1	0.103	0.250	0.650
Booster #3	0.750-0.800	0.260	0.497

IgM antibodies specific to HIV-1 were measured at Booster #3 and were found to be below cut-off (OD=0.113). Thus, IgM was below cut-off by day 42.

For purposes of this Example, IgG was measured using an HIV-1 Ab-IgG kit from ElectroNucleonics Inc. (ENI) in accordance with the manufacturer's instructions. IgM was measured using a modification of the HIV-1 ENI test kit. The IgM was measured using the method described in Example 1

EXAMPLE 3

Porcine Immune IgM Against HTLV-I

A 60 lb mixed Yorkshire breed pig was immunized (by subcutaneous injections in the left side of the neck) with 100 ug of HTLV-I viral lysate solubilized with Triton X-100 in 1 ml of PBS and 1 ml of Freund's adjuvant. No booster injections were administered. Ear vein blood samples were drawn prior to vaccination, 12 days and 17 days after vaccination. The pig was sacrificed at day 17 and its blood collected. The serum was separated, filter sterilized and frozen. Upon testing the serum showed the presence of IgM specific anti-HTLV-I at a level sufficient for use as a positive control. IgG was below detectable levels.

For purposes of this example IgG was measured using an HTLV-I Ab test kit from DuPont. The anti-IgG conjugate in the DuPont Test Kit is alkaline phosphatase (the substrate is supplied with the test kit). IgM was measured using a modification of the HTLV-I DuPont Test Kit in which the anti-IgG conjugate was substituted by an anti-IgM alkaline phosphatase used at a 1:10 dilution (Calbiochem, Catalog #401902).

The following results were obtained:

	IgM*	IgG*
Baseline		
Day 12	1.283	0.030
Day 17	2.200	0.300

*The cut-off had an OD value of 0.522 and the IgG positive control had an OD value of 0.979.

EXAMPLE 4

Procine Immune Against HIV-1

A 60 lb mixed Yorkshire pig was vaccinated with 50 ug of HIV-1 viral lysate as previously described in Example 1. The viral lysate was purified by sucrose gradient centrifugation at approximately 100,000× g (4° C. for one hour) on an ultracentrifuge.

Blood samples were drawn prior to the first immunization 13 days and 15 days after vaccination. No booster immunizations were required. The pig was sacrificed at

day 15. The blood samples collected were tested for IgG specific anti-HIV-1 proteins using an HIV-1 Antibody ELISA available from ElectroNucleonics (ENI). The blood samples were also tested for the presence of IgM using a modified ENI test kit. The modification comprised substituting affinity purified goat anti-human IgM, μ chain specific, labelled with horseradish peroxidase (available from Calbiochem, Catalog #401905) for the anti-IgG conjugate normally used. The substrate supplied with the ENI kit was used in accordance with the manufacturer's instructions. The incubation times and washing procedure were performed as directed in the ENI package insert. The results were read on a Behring ELISA processor at 492 nm with a 650 nm reference.

The results were as follows:

	Optical Density*	
	IgG	IgM
Baseline	0.042	0.085
Day 13	0.051	0.623
Day 15 (after sacrifice)	0.053	0.796

*The cut-off value was OD 0.192; the IgG positive control value was OD 0.921.

The results show that the collected blood had a high IgM antibody titer but was not contaminated by IgG specific anti-HIV-1, and therefore is useful as a positive control in an assay for detecting IgM specific anti-HIV-1.

In producing porcine immune IgM specific antibody, it has been found that an antibody titer within the optimum range is obtainable within 14–40 days. It is presently believed that initial vaccination, without subsequent booster injections, is sufficient to obtain the desired result. However, it should be understood that one or more boosters may be required depending on the animal immunized. The need for any such boosters can be determined by carefully monitoring levels of both IgM and IgG. Such monitoring is also necessary to insure obtaining blood containing sufficiently high levels of IgM but undetectable levels of IgG.

While preferred embodiments of the invention have been described, it will be apparent to those of ordinary skill in the art that various changes and modifications may be made therein without departing from the spirit and scope of the invention. Accordingly, the above description should be construed as illustrative, and not in a limiting sense, the scope of the invention being defined by the following claims.

I claim:

1. In an assay for detecting the presence or absence of a human antibody which binds to an antigen of a virus or a bacteria, comprising the steps of:

(a) contacting a sample of the antigen with a test sample of human biological fluid for times and under conditions sufficient for the antigen and any antibody present in the human biological fluid to form antigen-antibody complexes;

(b) at the same time contacting another sample of the antigen with a positive control comprising a procine antibody to the antigen under the same times and conditions as in step (a), said times and conditions being sufficient for the antigen and the non-human antibody in the positive control to form antigen-antibody complexes; and

(c) detecting the formation of any antigen-antibody complexes in steps (a) and (b) by simultaneously contacting an anti-human antibody with any antigen-antibody complex as formed, for times and under conditions sufficient for any antigen-antibody complexes formed in step (a) and step (b) to react with the anti-human antibody.

2. The assay of claim 1, wherein the non-human immune antibody comprises IgG, IgM or mixtures thereof.

3. The assay of claim 1, wherein the non-human immune antibody is an antibody to a human retrovirus indicative of acquired immune deficiency syndrome (AIDS) or AIDS related complex (ARC).

4. The assay of claim 3, wherein the non-human immune antibody comprises IgG, IgM or mixtures thereof.

5. The assay of claim 4, wherein the non-human immune antibody is a procine immune antibody.

6. The assay of claim 1, wherein the non-human immune antibody is an antibody to HTLV-I.

7. The assay of claim 6, wherein the non-human immune antibody is a porcine immune antibody.

8. The assay of claim 6, wherein the non-human immune antibody comprises IgG, IgM or mixtures thereof.

9. The assay of claim 1, wherein the non-human immune antibody is an antibody to cytomegalovirus (CMV).

10. The assay of claim 9, wherein the non-human immune antibody is a porcine immune antibody.

11. The assay of claim 9, wherein the non-human immune antibody comprises IgG, IgM or mixtures thereof.

12. The assay of claim 1, wherein the virus is selected from the group consisting of HIV-1, HIV-2, HTLV-I, HTLV-II, cytomegalovirus, and Epstein Barr virus.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,008,183 Page 1 of 2

DATED : April 16, 1991

INVENTOR(S) : KURT B. OSTHER

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

The title page showing the illustrative figure should be deleted to be replaced with the attached title page.

Column 1, line 35: Change "activitY to --activity--.

Column 1, line 58 Change "tYpe" to --type--.

Column 8, lines 10-11 Change "procine" to --porcine--.

Column 8, line 34 Change "procine" to --porcine--.

Signed and Sealed this

Ninth Day of February, 1993

Attest:

Attesting Officer

STEPHEN G. KUNIN

Acting Commissioner of Patents and Trademarks

United States Patent [19]

Osther

[11] Patent Number: 5,008,183

[45] Date of Patent: Apr. 16, 1991

[54] ASSAY SYSTEM FOR DETECTING ANTIBODY AND A METHOD OF PRODUCING NON-HUMAN IMMUNE ANTIBODY

[75] Inventor: Kurt B. Osther, Dallas, Tex.

[73] Assignee: Bio-Research Laboratories, Inc., Dallas, Tex.

[21] Appl. No.: 192,241

[22] Filed: May 10, 1988

[51] Int. Cl.[5] G01N 33/569

[52] U.S. Cl. 435/5; 435/7.32; 436/513; 436/532; 436/547; 436/815; 530/387; 530/830

[58] Field of Search 435/5, 7; 436/513, 532, 436/547, 815; 530/387, 830

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Primary Examiner—Christine Nucker

Attorney, Agent, or Firm—Bryan, Cave, McPheeters & McRoberts

[57] ABSTRACT

An improved assay method for detecting the presence of an antibody capable of binding with an antigen of a virus is provided. The improvement comprises using a non-human immune antibody which is reactive with an anti-human antibody as a positive control in the assay. Non-human immune IgM and a method of producing the IgM is also provided.

12 Claims, 1 Drawing Sheet

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Exhibit 9

Applicant E

United States Patent [19]

Jensen et al.

[11] **4,232,001**

[45] **Nov. 4, 1980**

[54] **METHODS AND MATERIALS FOR DETECTION OF ESTROPHILIN**

[75] Inventors: **Elwood V. Jensen; Eugene R. DeSombre, both of Chicago, Ill.**

[73] Assignee: **University Patents, Inc., Norwalk, Conn.**

[21] Appl. No.: **945,000**

[22] Filed: **Sep. 22, 1978**

[51] **Int. Cl.**[2] **G01N 33/16; A61K 43/00**

[52] **U.S. Cl.** ..... **424/1; 23/230 B; 260/112 B; 424/12**

[58] **Field of Search** ..... 424/1, 12; 23/230 B; 260/112 B

[56] References Cited

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Primary Examiner—Benjamin R. Padgett
Assistant Examiner—Christine M. Nucker
Attorney, Agent, or Firm—Merriam, Marshall & Bicknell

[57] **ABSTRACT**

Antibodies specifically immunologically reactive with estrophilin. Methods and materials for detection and quantification of estrophilin in tissue samples, notably those of human breast cancer tissue.

8 Claims, No Drawings

METHODS AND MATERIALS FOR DETECTION OF ESTROPHILIN

The invention described herein was made in the course of work under a grant or award from the Department of Health, Education and Welfare.

BACKGROUND

The present invention relates generally to antibodies for estrogen receptor protein and the in vitro detection and quantification of such protein through use of such antibodies. More specifically, the invention provides novel antibody preparations which have specific reactivity with estrogen recepetor and improved test methods and reagents for detection and quantification of receptor protein by means of immunological reactions.

It is generally recognized that specific estrogen-binding proteins, called "estrogen receptors" or, generically, "estrophilin" are responsible for the uptake of estrogenic hormones by certain tissues. The hormones are believed to interact with extranuclear estrophilin present in hormone dependent or "target" cells, with the "activated" estrogen-receptor complex so formed being translocated to the cell nucleus where it binds to the chromatin and in some way enhances the ability of the nucleus to synthesize certain types of RNA.

It has been determined that certain tissues, notably certain human breast cancer tissues, are estrogenic hormone "dependent" in the sense that systemic deprivation of supportive estrogen will result in regression of tissue growth and cell proliferation. As one example of this dependence, bilateral adrenalectomy can effect striking remission of advanced breast cancer in postmenopausal women and similar remissions are observed after hypophysectomy. Estrogen deprivation by surgical ablation of tissue responsible for estrogen production and/or endocrine additive therapy afford the most effective treatments presently available for advanced breast cancer. Unfortunately, less than one-half of the premenopausal patients and even a smaller fraction of postmenopausal patients respond to this type of therapy—indicating that breast cancer tissue is not always of the cellular type which is estrogenic hormone dependent. Consequently, it is significant to the prognosis and treatment of human breast cancer to be able to ascertain whether excised tumor tissue of a breast cancer patient is comprised predominantly of estrogen dependent cell types. On the basis of such information, a reasonable ablation response prediction may be made. Surgicial removal of estrogen producing glands for the purpose of estrogen deprivation may be restricted to those patients most likely to be helped by the procedure. Correlatively, other breast cancer patients can be spared the trauma of essentially useless surgery and may be placed immediately into alternative therapeutic programs such as radiation or chemotherapy.

Heretofore, the presence of estrogen dependent tissue in mammary tumor samples has principally been determined by quantitative detection of estrophilin in the sample through radiochemical assay. According to one such procedure developed by the inventors and their co-workers, radioactive (e.g., tritiated) estradiol is added to the cytosol—or supernatant fraction—of a homogenized tissue sample, and the tritiated estradiol reversibly combines with any estradiol-receptor protein present in the cytosol. The specimen is then subjected to low-salt, sucrose density gradient ultra-centrifugation and the protein-estradiol complex, being a large molecule, sediments with a characteristic velocity. A radioactive count can be used to quantify the complex. [See, e.g., Jensen, et al., J. Steroid Biochem., 7, 911–917 (1976); see also, Jensen, et al., "Estrogen Receptors and Breast Cancer Response to Adrenalectomy," National Cancer Institute Monograph, 34, 55–77 (1971)]. This procedure is carried out in the presence and in the absence of an inhibitor of the desired specific binding in order to identify and exclude any binding that is non-specific. The above analytical technique requires use of rather sophisticated, costly and uncommon ultracentrifugation apparatus, the operation of which requires a high degree of skill on the part of the laboratory worker. Other methods employed for receptor assays have similar limitations. [See, e.g., Korenman et al., J. Clin. Endocrinol. & Metab. 30, 699–645 (1970)] As a result, despite the exceptional usefulness of quantitative detection of estrophilin in prediction of response to endocrine therapy, the utilization of prior radiochemical assays is limited by scientific, geographic, and economic considerations.

It has long been recognized that immunochemical techniques for estrophilin detection would, if available, provide a simpler and less costly analytical procedure which would be susceptible to more widespread clinical use. Although suggestive evidence for the presence of antibodies to estrogen receptor in the serum of animals injected with partially purified estrophilin preparations has been reported [See, e.g., Soloff, et al., Biochem. Biophys. Res. Comm., 34, 141–147 (1969); Fox, et al., FEBS Lett., 63, 71–76 (1976); and Jensen, et al., Arch. Anat. Microscop. Morph. Exptl., 56 Suppl., 547–569 (1967)], the art has heretofore not been provided with any definitive demonstration that antibodies to estrophilin can be generated, prepared in quantity, and effectively employed in an assay for estrophilin—especially in tissue of differing species. Indeed the expectancy for success in the search for antibodies to estrophilin has been substantially diminished by past failure of allegedly highly purified progesterone receptor from oviduct tissue to give any trace of immune response in rabbits. Such findings have been supportive of wholely non-immunogenic characteristics for the proteinaceous steroid hormone receptors generally, and for estrophilin in particular.

BRIEF SUMMARY

According to the present invention there is provided for the first time a high yield of well-defined antibody isolate to estrophilin. More specifically, immunoglobulin obtained from the serum of animals immunized with a highly purified preparation of estradiol-receptor complex from calf uterine nuclei has been demonstrated to contain specific anti-estrophilin by five criteria: (1) precipitation of radioactive estradiol-recetor complex upon addition of heterologous species antibody (against the immunoglobulin of the immunized animal) to a mixture of the tritiated estradiol-receptor complex and the immunoglobulin; (2) adsorption of the estradiol-receptor complex by the immunoglobulin linked to a supporting polymer; (3) adsorption of the estradiol-receptor complex in the presence of the immunoglobulin by *Staphylococcus aureus* protein-A linked to a supporting polymer; (4) the ability of the immunoglobulin to increase the sedimentation rate of the estradiol-receptor complex; and (5) the ability of the immunoglobulin to modify the elution characteristics of estrophilin on gel filtration.

The purified preparation of estradiol/estrophilin complex employed for immunization according to the present invention is obtained by ammonium sulfate precipitation, gel filtration, and electrophoretic separation.

A further aspect of the present invention comprises a novel immunochemical procedure for quantitative detection of estrophilin in small amounts of tissue specimen, notably breast cancer tissue, through use of the above-mentioned antibody preparations. More specifically, according to procedures of the invention specific anti-estrophilin of the invention are cross-reacted with a preparation of body tissue such as mammary tumor tissue which is suspected to contain estrophilin in relatively large quantities. The reaction product so obtained is subjected to chemical, physical or radiological examination capable of quantifying the estrophilin in the sample. One preferred procedure involves treatment of a body tissue with, e.g., tritiated estradiol to form an estradiol/estrophilin complex, followed by contact with an antibody supporting or containing reagent. The quantity of estrophilin in the sample is determined by analysis of the immunologically-bound complex of estradiol/estrophilin/anti-estrophilin through scintillation counting or the like. According to another procedure, immunologically inert, but chemically or radiologically marked, particles have surface portions sensitized with the antibody preparation and are contacted with the tissue sample, with estrophilin quantified on the basis of agglutination phenomena. Agglutination phenomena of sensitized particles in contact with an estrophilin-containing specimen may also be monitored by other techniques (e.g., turbidometric techniques). Immunohistochemical procedures involving fluorescent or peroxidase coupled anti-estrophilin preparations are also made available by the invention.

Reagents prepared according to the invention include immunologically inert particles, surface portions of which are sensitized with specific antibody preparations of the invention.

Further aspects and advantages of the invention will become apparent to those skilled in the art upon consideration of the following detailed description of presently preferred embodiments thereof.

DETAILED DESCRIPTION

The following description includes use of certain abbreviations: "E*" shall mean tritiated estradiol; "R" shall mean estrophilin; "E*R" or "E*R complex" shall designate the radioactive complex of estradiol and estrophilin; "Ig-i" shall designate the immunoglobin fraction obtained from serum of an animal immunized with E*R; and "Ig-n" shall designate immunoglobin from serum of a nonimmunized animal.

The reagents employed in certain of the illustrative examples include the following. [6,7-^{3}H] Estradiol-17β (57 Ci/mmol) and [2,4,6,7-^{3}H]estradiol-17β(108 Ci/mmol) were obtained from New England Nuclear Co. and, unless otherwise noted, E* represents the 6,7-tritiated hormone. Tubercle bacilli and Freund's complete and incomplete adjuvants were purchased from Difco, bordetella pertussis vaccine from Eli Lilly & Co., and *Staphylococcus aureus* protein-A bound to Sepharose CL-4B from Pharmacia. Immunoglobulin from immunized and nonimmunized rabbits was coupled to cyanogen bromide-activated Sepharose 4B by the procedure of Cuatrecasas, et al., "Affinity Chromatography" in *Methods in Enzymology*, (Academic Press, New York) Vol. 22, pp. 345-378 (1971). Antiserum to rabbit Ig was prepared by immunizing a female goat with purified rabbit immunoglobulin (12.5 mg for primary injection; 5-12 mg for booster injections) in a manner similar to that described for immunization of rabbits with E*R except that the emulsion containing the antigen was injected subcutaneously. Unless otherwise noted, phosphate buffers were prepared from sodium salts, and all buffers contained 0.01% (wt/vol) sodium azide. Phosphate-buffered saline contained 150 mM sodium chloride in 10 mM phosphate, pH 7.8. Buffers using Tris (T), pH 7.4 at 23° sometimes containing potassium chloride (K) and disodium EDTA (E), are designated according to the millimolarity of their components; e.g., $T_{10}K_{400}E_{1.5}$ = 10 mM Tris/400 mM KCl/1.5 mM EDTA.

EXAMPLE 1

Preparation of Purified Estradiol-Receptor Complex (E*R)

The estradiol-receptor protein in the form of an estradiol-receptor complex which is capable of inducing formation of an antibody having a specific reactivity with the estradiol-receptor protein in an immunological reaction was prepared according to a modification of the method of Gorell, et al., "Purification of Nuclear Estrogen Receptors" in *Proceedings of the Fifth International Congress of Endocrinology*, (Excerpta Medica Foundation, Amsterdam) Vol. 1, pp. 467-472 (1977). Unless otherwise noted, all procedures were carried out at 0°-2° C.

A calf uterine homogenate in T_{10} buffer was centrifuged at 10,000×g to separate the cytosol from crude nuclear sediment. The cytosol was treated with 30 nM E* (5.7 Ci nmol.). One-fifth of the sediment was washed successively with $K_{400}T_{10}$, pH 7.9, and T_{10}, pH 7.5, and then resuspended in the cytosol. After incubation of the stirred mixture at 25° C. for 60 minutes, the nuclear sediment was collected by centrifugation, washed with T_{10} buffer and extracted with $K_{400}T_{10}$ buffer. The extracted receptor complex was precipitated with ammonium sulfate (30% of saturation) and redissolved in $K_{400}T_{25}$, pH 7.5, (0.1 volume of the original extract) by gentle homogenization followed by stirring for 20 minutes. After clarification by centrifugation at 98,000×g for 60 minutes, the solution was subjected to gel filtration on Sephadex G-200 (Regular grade, Pharmacia) in $K_{400}T_{25}$ buffer. The estrogen receptor complex (determined by radioactivity assay) separated from the bulk of other proteins (determined by optical density at 280 nm) which eluted in the void volume. The pooled receptor-containing fractions were concentrated first by ultrafiltration with XM-50 membrane (Amicon) and further by salt precipitation with ammonium sulfate (30% of saturation). The receptor complex was redissolved in $K_{30}T_{10}$, pH 7.5, and subjected to polyacryamide gel electrophoresis using 0.7 centimeter diameter, 5% or 7% acryamide gel with 2.5% cross-linkage prepared in Tris-HCl buffer pH 8.9. The electrophoresis is carried out with a current of 3 milliamps per gel, using electrode buffers of Tris-glycine, pH 8.3, or Tris-borate, pH 8.6 The receptor protein was isolated by slicing the gels, extracting the pooled gel slices with 10 nM sodium phosphate buffer, pH 7.4, (0.1 ml per slice) and combining the fractions with the highest radioactivity (R_f=0.45 for 7% gels; R_f=0.60 for 5% gels). The pooled extract was dialyzed against $K_{10}T_{10}$ (azide-free), pH 7.5, and lyophilized to dryness.

The product has an isoelectric point of about 6.0, shows a single stained protein band on acrylamide gel electrophoresis, and analytical ultracentrifugation indicates a single ultraviolet absorbing macromolecular species. Elution from a calibrated Sephadex G-200 column indicates a Stokes radius of 36.5, corresponding to a molecular weight of about 66,000 daltons.

EXAMPLE 2

Preparation of Antibodies for Estradiol Receptor Protein

Antibody preparations having specific reactivity with estrophilin protein are obtained by immunizing rabbits or like mammals with the purified E*R complex of Example 1 according to the procedure described by Vaitukaitis, et al., J. Clin. Endocrinol. Metab. 33, 988–991 (1971). For the primary immunization of six month-old male New Zealand White rabbits, an emulsion, prepared by homogenizing a saline solution containing 20 μg of the E*R with an equal volume of Freund's complete adjuvant and an additional 5 mg of dried tubercle bacilli, was injected intradermally at multiple sites on the back, while 0.5 ml of bordetella pertussis vaccine without added antigen was injected in the thigh. Six booster injections were given over a period of one year with an emulsion of the immunogen and Freund's incomplete adjuvant; the last two booster injections, containing 20 and 50 μg of receptor, respectively, were given about 45 days, and 15 days before collecting the blood from the marginal ear vein of the rabbit. A crude immunoglobulin fraction (Ig-i) was prepared from the serum by precipitation with 33% saturated ammonium sulfate in 50 mM phosphate buffer, pH 7.4, according to the procedure of Shiu, et al., Biochem J., 157, pp. 619–625 (1976). The washed precipitate was redissolved in phosphate-buffered saline (or in some experiments in 20 mM phosphate, pH 7.4) and the dialyzed solution, after clarification by centrifugation, was analyzed for protein by its absorbance at 280 nm and for the absence of other serum proteins by acrylamide gel electrophoresis. Immunoglobulin (Ig-n), prepared similarly from serum of nonimmunized animals (as well as from the immunized animals before any antibody titer appeared), was used as a control.

EXAMPLE 3

Preparation of Hormone-Receptor Complexes

Estradiol-receptor complexes of uterine cytosols from immature calves, rats, mice, and guinea pigs were prepared by homogenizing the tissues in four volumes of T_{10} buffer, using a Polytron PT-10 homogenizer with efficient cooling, and making the high-speed supernatant fraction 20 nM in E*, after 60 minutes at 4° the excess E* was removed with dextran-coated charcoal. A complex of human breast cancer cytosol was prepared similarly except that the tumor specimen, pulverized while frozen in liquid nitrogen, was homogenized in T_{10} containing 0.5 mM dithiothreitol, and the cytosol fraction was made 0.5 nM in E* (108 Ci/mmol) without the use of charcoal. Crude nuclear complex of calf uterus was prepared by extraction of washed nuclear sediment with $T_{10}K_{400}$ after its incubation for 60 minutes at 25° with 20 nM E* in calf uterine cytosol. Rat nuclear complex was obtained by similar extraction of the nuclear sediment from a homogenate in T_{10} of immature rat uteri excised 4 hours after the subcutaneous injection of 100 ng (20.8 μCi) of E* in 0.2 ml of saline.

EXAMPLE 4

Immunochemical Interaction Studies

For double antibody precipitation of the crude estradiol-receptor complexes, a solution of E*R (1.0 pmol), normal rabbit serum (10 μl), and immunized rabbit serum (10 μl) in 1.0 ml of phosphate-buffered saline containing 10 mM EDTA was incubated at 4° for four hours, after which sufficient goat antiserum against rabbit Ig was added to precipitate all the rabbit Ig. Controls used 20 μl of normal rabbit serum and no immunized rabbit serum. After 16 hours the mixtures were centrifuged and the pellets were dissolved in 100 μl of 0.1 M NaOH; radioactivity was measured in 10 ml of scintillation mixture containing 10 mM HCl.

For immunoadsorption experiments, a solution of 0.8 pmol of E*R or E* was incubated for four hours at 4° with an agitated suspension of either Sepharose-Ig-i or Sepharose-Ig-n (containing 300 μg of protein) or of Sepharose alone in a total volume of 600 μl of $T_{10}K_{400}$. Similarly, 0.3-pmol aliquots of E*R or E* in $T_{10}K_{400}$ were incubated at 4° for 90 minutes with 100 μg of either Ig-i or Ig-n (or with no added immunoglobulin) and then for four hours longer with an agitated suspension of 50 μl of Sepharose-protein-A in a final volume of 500 μl of $T_{10}K_{400}$. After centrifugation, the beads were washed and the combined supernatant and washings were assayed for radioactivity in Triton X-100 scintillation mixture.

EXAMPLE 5

Sedimentation Studies

Various hormone-receptor complexes (0.05–2 pmol in 150 μl of T_{10} cytosol or $T_{10}K_{400}$ nuclear extract) and Ig-i or Ign-n (usually 200 μg) were incubated at 4° for 1–5 hours in a final volume of 220 μl of T_{10} or $T_{10}K_{400}$ depending on the gradient to be used. A 200-μl uliquot of each mixture was layered on 3.5 ml of a 10–30% sucrose gradient containing either $T_{10}K_{10}E_{1.5}$ (low salt) or $T_{10}K_{400}E_{1.5}$ (high salt) and centrifuged at 2° for 16 hours at 253,000×g. Successive 100-μl fractions were collected from the bottom and radioactivity was measured in Triton X-100 scintillation mixture. In some studies bovine plasma albumin (4.6 S), bovine gamma globulin (7.0 S), βamylase (9.2 S), and catalase (11.3 S) were sedimented in parallel gradients to serve as markers.

EXAMPLE 6

Gel Filtration Studies

Gel filtration studies were carried out with the calcium-stabilized form of the cytosol estrogen receptor complex obtained by homogenizing calf uterus in $T_{10}E_{1.5}$ and incubating the cytosol fraction with 1 M KCl and 4 mM $CaCl_2$, with or without 20 nM E*, for 60 minutes at 4° C. After precipitation with ammonium sulfate (25% of saturation) the receptor (R) or estrogen-receptor complex (E*R) was dissolved in $T_{10}K_{400}$ and clarified by centrifugation at 250,000×g for 30 minutes.

For gel filtration experiments aliquots of E*R or R (5 pmol) and Ig-n or Ig-i (1.0 mg) were incubated for 60 minutes at 4° C. and then filtered through Sephadex G-200 columns (0.9×55 cm) equilibrated in $T_{10}K_{400}$. Fractions (0.3 ml) were collected and either assayed for

radioactivity or (in the case of materials derived from cytosol fractions not incubated with E*) treated with E* and then dextran coated charcoal to remove excess E*. The latter procedure resulted in a material then assayed for radioactivity. The void volume of the column was determined with blue dextran. Bovine serum albumin and rabbit immunoglobulin were employed as markers.

The results of the above-described immunochemical, sedimentation and gel filtration studies demonstrated that immunoglobulin from the serum of a rabbit immunized with purified estrogen receptor complex of calf uterine nuclei contains specific antibodies to the estrophilin receptor protein. As shown in the accompanying Table 1, in the presence of Ig-i, but not of Ig-n, a significant amount of tritiated estradiol in the form of E*R complex is precipitated by antiserum to rabbit gamma globulin or bound to *Staphylococcus aureus protein-A, a substance that reacts specifically with the IgG type of antibody. Simtlarly, Ig-i linked to Sepharose binds a much greater proportion of tritiated estradiol/estrophilin (E*R) complex than does Sepharose-Ig-n or Sepharose alone.*

TABLE 1

Interaction of rabbit Ig with E*R complexes of calf uterus				
	Form of	% E* precipitated or bound		
Method	Estradiol	Ig-i	Ig-n	NoIG
Double antibody precipitation	E*R nuclear	61	10	
	E*R cytosol	56	3	
	E*	2	2	
Binding to Sepharose-Ig	E*R nuclear	66	21	17
	E*	6	15	7
Binding to Sepharose-Protein-A	E*R nuclear	70	17	30
	E*	3	5	3

From sedimentation studies, it was found that addition of Ig-i, but not of Ig-n, causes an increase in the sedimentation velocity of the purified 4.8 S E*R complex used as the antigen in the immunization.

Sedimentation patterns reveal that the above-described antibody preparation derived through use of estrophilin from calf uterus is cross reactive with estrogen receptor complexes from rat, mouse, and guinea pig uteri and from human breast cancer. Significantly, however, the failure of the anti-estrophilin preparations to react with the dihydrotestosterone-receptor of rat prostate or the progesterone receptor of chick oviduct, shows a specificity of the antibody for estrophilin protein.

Gel filtration studies verified that Ig-i but not Ig-n reacts with both the E*R and R forms of estrogen receptors as demonstrated by a shift of elution to the void volume on the column (Sephadex G-200). For the uncomplexed receptor, the eluted R/IG-i complex could be labelled with E* to form E*/R/Ig-i, demonstrating that the complexing of the estradiol hormone with the receptor does not block the binding site of estrophilin.

In sum, immunoglobulin obtained from the serum of rabbits immunized with a highly purified preparation of E*R complex from calf uterine nuclei is shown to contain specific antibodies to estrophilin by five criteria: (1) precipitation of the radioactive steroid upon addition of goat antibody against rabbit immunoglobulin to a mixture of the tritiated estradiol-receptor complex and the immunoglobulin, (2) adsorption of the estradiol-receptor complex by the immunoglobulin linked to a polymer support, e.g., Sepharose, (3) adsorption of the estradiol-receptor complex in the presence of the immunoglubulin by *Staphylococcus aureus protein-A linked to a polymer support, e.g., Sepharose;* (4) the ability of the immunoglubulin to increase the sedimentation rate of the estradiol-receptor complex; and (5) the ability of the immunoglobulin to modify elution characteristics of estrophilin on gel filtration. The antibodies cross-react with the nuclear receptor of calf, rabbit, sheep and rat uterus, as well as with the extranuclear receptor of calf, rat, mouse, guinea pig, sheep, and rabbit uterus. Cross-reactivity is also observed with nuclear receptor of MCF-7 (human breast cell) and rat endometrial tumors as well as with extranuclear receptor of rat breast, human breast, MCF-7 breast, rat endometrial, and rat pituitary tumors.

The antibodies do not react with either the nuclear or extranuclear dihydrotestosterone-receptor complexes of rat prostate or with the extrauclear progesterone-receptor complex of chick oviduct, rabbit uterus and rat endometrial tumor.

The cross-reactivity of the anti-estrophilin (Ig-i) with estrophilin from human breast cancer makes possible the use of simple immunoassays including radioimmunoassays for the estradiol-receptor protein content of breast cancer tissue as a guide to endocrine therapy.

The reaction of anti-estrophilin prepared according to the invention with uncomplexed receptor (R) is indicated by the ability of R to compete with E*R for a limiting amount of antibody. This type of analysis revealed that, whether complexed with estradiol or not, calcium-stabilized estrophilin from calf uterine cytosol shows essentially identical competition with radioactive cytosol receptor complex for the antibody, although somewhat less than that shown by receptor from rat endometrial tumor. On the basis of this finding, a simple immunoassay for estrophilin in extracts of tissue homogenates is available—limited only by the relatively low radioactivity of the tritiated estradiol marker. Some increase in sensitivity may be gained through use of hexatritiated estradiol and, of course, radiodinated receptor and/or antibody would be most useful in such an assay.

According to the invention novel immunological reagents are provided when the immunoglobulin fraction of serum of an animal immunized with purified estrophilin estradiol complex is employed, with or without prior treatment to remove non-specific antibodies in the immunoglobulin, to sensitize immunologically inert particulate materials such as stabilized erythrocytes (e.g., prepared according to the procedures of U.S. Pat. Nos. 3,714,345; 3,715,427; and/or 3,924,541), bentonite, collodium, crystalline cholesterol, quartz, synthetic resins, various kinds of synthetic latex (see, e.g., U.S. Pat. No. 3,551,555), or liposomes prepared from phospholipids and sterols including radioactive material-containing or free radical-containing liposomes. Such sensitized particles are useful when employed in direct agglutination assays wherein estrophilin or estrophilin radioisotopic estradiol complex in a tissue sample will be bound to and effect agglutination of the particles, allowing quantification of the complex by standard radioimmunoassay techniques. Alternatively, when antibody materials are employed to sensitize radioactive material or free radical-containing particles, estrophilin content of a tissue sample homogenizate may be determined by adding such particles to the sample, withdrawing particles agglutinated by the estrophilin and "counting" the particles as aggregated. Further, tech-

niques using radiochemically-labelled, or enzyme-linked, or otherwise detectable anti-estrophilin may provide the basis for estrophilin assays that do not detect the complex formed by binding with estradiol (E*). Substantial advantages are expected to accompany procedures which are independent of the reversible binding of estrophilin in the sample to radioactive estradiol. In particular, such procedures should be able to detect the amount of free estrophilin as well as estrophilin already associated with non-radioactive, endogeneous estradiol.

In one such further application, anti-estrophilin provides the basis for a specific immunohistochemical procedure for the detection and quantification of estrogen receptor in pathologic sections of cancer tissue. Such sections are incubated with solutions of the antibody and, after washing with buffer, incubated either with fluorescent-labelled second antibody (to the immunoglobulin) or with peroxidase-antiperoxidase-complexed second antibody. After further washing, the sections are examined for the presence of estrophilin by fluorescent microscopy, or after peroxidase staining, by light microscopy. This technique allows the detection of estrophilin in tissue sections carried out in the same surgicial pahological laboratory where the cancer diagnosis is made.

Numerous modifications and variations of the invention are expected to occur to those skilled in the art upon consideration of the foregoing detailed description. While the above-described preferred embodiment of procedures for preparing and isolating anti-estrophilin represents the best mode presently available for effecting isolation of the antibody substances in their purest form, other techniques are certainly within the contemplation of the invention. As one example, lymph node cells from rabbits immunized with the purified E*R complex may be fused with mouse myeloma cells in the presence of polyethylene glycol, generally according to the technique of Milstein [Kohler and Milstein, 256, 495–497 (1975)] The surviving hybrids would then be screened by selective cloning to obtain only cells which produce the anti-estrophilin antibody substance. The cell line(s) so obtained would be grown in suspension culture to provide substantial quantities of anti-estrophilin which, when separated from contaminating myeloma antibody by immuno-absorption, may be employed as noted above in immunoassay techniques for quantitative detection of estrophilin in a tissue sample.

The availability of monospecific anti-estrophilin provided by the invention is expected to make possible further developments is diagnosis of breast cancer and other cancer tissues which contain estrophilin. Linkage of an isotope, (e.g., Iodine) or other radiologically detectable moiety to the specific anti-estrophilin would provide a basis for a new diagnostic scanning technique. After administration to a patient having an estrophilin-rich primary tumor, such a reagent has the potential to concentrate in a metastatic tumor site also containing estrophilin, causing metastases to be evident on an appropriate radiologic body scan.

Furthermore, since tumor components or their partial degradation components are often released into the blood, an assay for immunoreactive fragments of estrophilin in the sera of patients with (or suspected of having) an estrophilin-containing cancer could provide a means for early diagnosis of metastatic disease.

Consequently, only such limitations as appear in the appended claims should be placed upon the invention as above described.

What is claimed is:

1. A specific, immunologically active anti-estrophilin antibody obtained as a serum immunoglobulin component from an animal immunized with estrophilin, and characterized by its donation to the immunoglobulin of the following properties;

(1) the ability to precipitate estrophilin from a mixture of estrophilin and said immunoglobulin upon addition of heterologous species antibodies to the immunoglobulin of the serum donor species;

(2) the ability to mediate rapid absorption of estrophilin from a fluid by support polymer when said immunoglobulin is linked to the support polymer;

(3) the ability of said immunoglobulin to mediate absorption of estrophilin from a fluid by *S. aureus* protein-A linked to a support polymer;

(4) the ability of said immunoglobulin to increase the sedimentation rate of estrophilin; and,

(5) the ability of said immunoglobulin to modify elution characteristics of estrophilin on gel filtration.

2. An immunological reagent comprising the anti-estrophilin antibody of claim 1 in combination with a radiologically detectable marker substance.

3. The reagent of claim 2 wherein the marker substance is tritiated estradiol.

4. An immunological reagent comprising the anti-estrophilin antibody of claim 1 and a plurality of immunologically inert particles, said substance providing an immunologically sensitive surface on said particles.

5. An immunological assay for estrophilin in a tissue sample said assay comprising contacting said sample with the reagent of claim 4 and quantitatively detecting the presence of estrophilin by monitoring agglutination behavior of said reagent particles.

6. An assay according to claim 5 wherein said tissue sample is a human breast cancer tissue sample.

7. The method for obtaining serum immunoglobin containing a specific anti-estrophilin antibody, said method comprising the steps of:

preparing a purified complex of radioisotopic estradiol and estrophilin by incubating estrophilin-containing tissue in the presence of radioisotopic estradiol and isolating the complex from said incubate by precipitation, gel filtration and electrophoresis;

inoculating an immunologically active animal with said complex and,

isolating serum immunoglobin from said animal.

8. Anti-estrophilin antibody raised in tissue of animal origin against estrophilin of animal origin, said antibody characterized by being immunologically reactive with estrophilin of human breast cancer tissue.

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Exhibit 10

1515

Tumor inflammatory response induced by immunization with autologous melanoma cells conjugated to dinitrophenol(DNP). D. Berd, M.J. Mastrangelo, C. Green, C. Clark, and E. Hart. Thomas Jefferson University, Philadelphia, PA 19107.

Treatment of melanoma patients with an autologous vaccine preceded by low dose cyclophosphamide (CY) induces delayed-type hypersensitivity (DTH) to melanoma cells, and in some cases, regression of metastatic tumors. Now, we are attempting to increase the efficiency of the process by immunizing with tumor cells conjugated to the hapten, DNP. Patients with metastatic melanoma were sensitized to DNP by topical application of dinitrochlorobenzene (DNCB). Two weeks later, they were injected with a vaccine consisting of 10-25x10(6) autologous, irradiated melanoma cells conjugated to DNP and mixed with BCG. CY 300 mg/M^2 IV was given 3 days before DNCB or vaccine. Of 4 patients evaluable so far, 3 have developed a striking inflammatory response in tumor masses after 2 vaccine treatments (8 weeks). Patient #1 developed erythema and swelling in the >50 large (1-3 cm) dermal metastases on her leg and lower abdomen, followed by ulceration and drainage of necrotic material, and some are beginning to regress. Biopsy showed infiltration with CD4+ and CD8+ T lymphocytes. Patient #2 developed erythema and swelling in the skin of her lower abdomen and groin overlying large (8 cm) nodal masses. These have not yet regressed, but have changed in consistency from rock-hard to fluctuant. Patient #3 exhibited moderate erythema in the skin overlying subcutaneous metastases. All 3 patients have developed DTH to both DNCB and to DNP-conjugated autologous lymphocytes. Although these results are preliminary, they suggest that this new strategy may represent a significant advance in the immunotherapy of human melanoma.

1516

Inhibition of Tumor-Induced Suppressor T Lymphocyte (Ts) Activity by Murine Interferon Beta (IFN-B). Deepak M. Sahasrabudhe, University of Rochester Cancer Center, Rochester, NY, 14642

In some tumor models inhibition of Ts-activity is a prerequisite to successful immunotherapy. Based on our data in the DNFB model (J Exp Med 166:1573, 1987) the effect of IFN-B on P815 mastocytoma-induced Ts-activity was evaluated.

In this model, concomitant antitumor immunity (Tc) peaks by Day 10 and is down regulated by Ts by Day 15. Cytotoxicity generated after a mixed lymphocyte tumor culture (MLTC) correlates with in vivo immunity and suppression of cytotoxicity correlates with in vivo Ts-activity.

Tumors were initiated by injecting 2 x 10^6 P815 cells subcutaneously on Day 1. IFN-B (10U, 1000U, 5000U) or buffer were injected i.v. every other day x 5 doses starting on Day 5. On Day 16, MLTC's were set up. Five days later a cytotoxicity assay was performed against ^{51}Cr labelled P815 cells. % specific lysis is shown. Numbers in parenthesis represent the dose of IFN-B.

	Tc +		Tc		Ts	Tc +Ts	Ts	Tc +Ts	Ts	Tc +Ts
E:T	Tc	Naive	Ts	+Ts	(10)	(10)	(1000)	(1000)	(5000)	(5000)
50:1	88	81	0	19	6	22	23	20	81	84
25:1	84	76	0	12	2	21	1	21	63	75
12:1	78	79	2	15	3	24	6	23	58	81
6:1	70	69	1	7	0	9	0	20	38	64
3:1	56	55	0	8	1	13	0	12	21	48

Treatment with IFN-B 5000U every other day x 6 doses abrogated Ts-activity without adversely affecting cytotoxicity. IFN-B may be a useful adjunct in the immunotherapy of selected tumors.

1517

Anti-idiotype monoclonal antibody immunization therapy of cutaneous T cell lymphoma. Chatterjee, M., Foon, K., Seon, B.K., Barcos, M. and Kohler, H., Roswell Park Mem. Inst., Buffalo, NY 14263, and UCSD, San Diego, CA 92161.

Cutaneous T cell lymphoma (CTCL) is an indolent non-Hodgkin's lymphoma which is not cured by standard therapies once it reaches advanced stage. A novel approach to therapy is to use internal image anti-idiotype (Id) mAbs as antigen (Ag) substitute for the induction of immunity. We have generated anti-Id mAb (Ab2) binding to a hybridoma SN2 (Ab1), which recognizes a unique glycoprotein, gp37, expressed by a subset of human leukemic T cells (J. Immunol. 139:1354, 1987). At least 2 of these Ab2 may indeed carry the internal image of the gp37 Ag (J. Immunol. 141:1398, 1988). Recently, we investigated the distribution of gp37 Ag by a sensitive immunoperoxidase staining method using mAb SN2. SN2 had a high specificity for T-leukemia/lymphoma cells and did not react with any normal adult tissues tested including thymus, lymphocytes, bone marrow cells, spleen, liver, kidney, lung, brain, heart, etc. CTCL cells from 5/4 out of 6 patients were strongly positive for gp37 Ag with intense surface membrane staining. The binding of radiolabeled SN2 to CTCL cells was studied for inhibition in the presence of the anti-Id mAbs 4EA2 and 4DC6 which mimic the gp37 Ag. Both clones inhibited the binding 100% and 80% respectively at a concentration of 50 ng. We also generated a murine Ab3 mAb (anti-anti-Id) by immunizing mice with these anti-Id mAb (Ab2). This Ab3 mAb reacts with CTCL cells in an identical fashion as the original Ab1 (SN2). Collectively, these data suggest that Ab2 4EA2 and 4DC6 may be useful for active immunotherapy of CTCL patients. We also plan to study the CTCL patients in a phase I clinical trial to determine the effects of this type of therapy on various components of the immune system (both humoral and cellular) and try to identify the criteria to select patients who may benefit from anti-idiotype vaccine therapy.

1518

Syngeneic murine monoclonal antiidiotypes bearing the internal image of a human breast cancer associated antigen. J. Schmitz and H. Ozer. The Dept. of Microbiology, S.U.N.Y. at Buffalo, Buffalo, NY 14214 and the Division of Medical Oncology, The Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

According to Jerne's network theory, some antiidiotypes (Ab2) mimic external antigens recognized by specific antibodies (Ab1) and may be used in place of antigen for immunization. The murine monoclonal antibody F36/22 (IgG3, κ), specific for ductal carcinoma antigen (DCA) was used to generate syngeneic monoclonal antiidiotypes bearing the internal image of DCA. Female BALB/c mice were inoculated intraperitoneally every other week with 100 μg of F36/22 coupled to keyhole limpet hemocyanin; the first time in complete Freund's adjuvant and subsequently in incomplete adjuvant. Splenic lymphocytes were fused with the murine cell line P3X63 Ag8.653 3 days after the fourth immunization using 50% polyethylene glycol (m.w. 3000). Two hybrids, MTO-1 and MTO-2, were selected based on the ability of culture supernatants to bind to F36/22 but not to the control antibody 2A31F6 (IgG3, κ) in an enzyme linked immunosorbent assay (ELISA) and cloned by limiting dilution. Paratope specificity of Ab2 was demonstrated in two ELISA assays. First, the binding of labeled F36/22 to DCA was inhibited 100% and 75% by 1.6 μg of MTO-2 and MTO-1 respectively. Second, the binding of labeled Ab2 to Ab1 was inhibited by purified DCA. MTO-1 neither enhances nor inhibits the binding of labeled MTO-2 to Ab1 although in the presence of MTO-2, binding of labeled MTO-1 is enhanced by 100% indicating that these Ab2 recognize distinct idiotopes. Rabbits immunized biweekly with MTO-1 or MTO-2 developed antibodies that bound specifically to DCA demonstrating that MTO-1 and MTO-2 bear the internal image of DCA. These data suggest that MTO-1 and MTO-2 could potentially be utilized to immunize high risk patients against progression or development of DCA positive tumors.

Exhibit 11

Immunology, 1970, 19, 189.

1766
7011

Lymphocyte Transformation in Contact Sensitivity

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Summary. Leucocyte suspensions containing 97–100 per cent lymphocytes were prepared from the peripheral blood of unsensitized guinea-pigs as well as animals previously sensitized with 1-chloro-2,4-dinitrobenzene. The lymphocytes were dinitrophenylated and cultured for 5 days *in vitro*. Transformed cells (8–22 per cent) and an increased (100–1000 fold) uptake of tritiated thymidine were observed only in cultures of lymphocytes from sensitized animals. Smaller numbers of transformed cells (1–2 per cent) were found in only a few cultures of sensitive lymphocytes incubated with either dinitrophenylated autologous erythrocytes or serum proteins. These results strongly suggest that transformation may specifically follow the conjugation of DNFB with live lymphocytes from sensitized donors.

INTRODUCTION

Halogenated dinitrobenzenes such as 1-chloro- and 1-fluoro-2,4-dinitrobenzene (DNCB and DNFB respectively) are commonly used to elicit delayed hypersensitivity (Bloom and Chase, 1967; Turk, 1967). The dermal reactions evoked by these substances in sensitized animals may result from an interaction between specifically sensitive cells and dinitrophenylated acellular skin components which act as antigens (Eisen and Tabachnick, 1958). However, it is possible that the highly reactive halogenated dinitrobenzenes (Eisen, Orris and Belman, 1952) may also react directly with lymphocytes, and subsequently initiate the dermal reaction by releasing substances such as the macrophage inhibiting factor (David, Al-Askari, Lawrence and Thomas, 1964; Bloom and Bennett, 1966) and the lymph node permeability factor of Willoughby, Boughton, Spector and Schild (1962). Since specific antigens can induce lymphocyte transformation *in vitro* Pearmain, Lycette and Fitzgerald, 1963; Elves, Roath and Israëls, 1963), a method is available for the determination of the ability of DNFB to act as a specific antigen by inducing transformation of sensitized, living lymphocytes. The occurrence of transformation of dinitrophenylated lymphocytes *in vitro* would suggest that DNFB can act directly on lymphocytes *in vivo*.

Furthermore, if delayed reactions are carrier-specific (Gell and Wolstencroft, 1967) the complete antigen which induces transformation of dinitrophenylated lymphocytes should contain a moiety supplied by the lymphocyte. It seems unlikely that a moiety with an identical molecular configuration can be supplied by acellular constituents of the skin. For the same reasons, it seems necessary to suppose that the antigen which initially induces hypersensitivity is also derived from dinitrophenylated lymphocytes formed during the sensitizing exposure to the chemical. An acquisition of information in this way would support a mechanism of peripheral sensitization suggested by Medawar (1958) and Strober and Gowans (1965). This paper reports the *in vitro* transformation of living, dinitrophenylated lymphocytes.

MATERIALS AND METHODS

Preparation of DNFB solutions

For conjugation with lymphocytes, erythrocytes and serum proteins, a stock solution of DNFB (Fluka A. G., Buchs, S. G.) was prepared by dissolving approximately 300 μg DNFB per ml in 0·2 M (pH 8·5) phosphate buffer at 60°. The actual concentration of DNFB in the buffer was determined spectrophotometrically.

For conjugation with bovine serum albumin (BSA; Commonwealth Serum Laboratories, Melbourne, Vic.) a DNFB solution was prepared in 0·2 M citrate–phosphate–borate buffer (Teorell and Stenhagen, 1938) at the appropriate pH.

Preparation of DNCB solutions

For sensitization, a stock solution of DNCB (British Drug Houses, England) in distilled water was prepared by dissolving approximately 300 μg DNCB per ml and the concentration of DNCB then adjusted to 40 μg/ml under spectrophotometric control. For testing, a stock solution of DNCB was prepared by dissolving a known quantity of DNCB in olive oil B.P. (N.S.W. Government Stores). The stock solution was diluted as required with a 1 : 9 mixture of olive oil and petroleum ether (B.P. 60°–80°, A.R. grade, B.D.H.).

Protein determinations were done according to the method of Lowry, Rosebrough, Farr and Randall (1951), using BSA as the standard.

Sensitization and skin testing

Outbred albino female guinea-pigs (600–800 g) were sensitized and tested according to Baumgarten and Wilhelm (1969). Briefly, the animals were sensitized by multiple injections of 40 μg DNCB in the nuchal skin. Eleven to 14 days after sensitization, skin testing was done by applying 20 μl DNCB solution (0·03, 0·1, 0·3 and 1·0 per cent) to each of eight randomly distributed sites on the closely shaved dorsal skin of the trunk using Hamilton 250 μl syringes (New Jersey, U.S.A.) fitted with blunted 26 G hypodermic needles. The solution was spread with a rounded glass rod in the direction of hair growth over a 1·5–2·0 cm^2 area. The lesions were examined after 20–24 hours for the presence of erythema; the animals were subsequently given intravenous Evans blue (1·2 ml/kg body weight, as a solution containing 25 mg dye per ml of 0·45 per cent saline) to demonstrate the development of increased vascular permeability (Voisin and Toullet, 1960).

Preparation of lymphocytes

Blood was usually obtained from guinea-pigs by cardiac puncture, occasionally by severing the jugular vein. It was placed in sterile bottles (containing 10–15 glass beads 3–5 mm diameter), defibrinated by gentle shaking for 5–10 minutes and transferred into fresh bottles. To each millilitre of blood was added 0·3 ml of 1 per cent methyl cellulose (U.S.P. Grade, Dow Chemicals Co.) solution in 0·2 M phosphate buffer, pH 7·2. After mixing by inversion, the blood was allowed to sediment for 45 minutes at 37°. The uppermost two-thirds of the top (leucocyte-rich) layer was removed and centrifuged at 400 *g* for 5 minutes. The supernatant was then separated and the cells washed twice with Hanks's solution (C.S.L., Melbourne, Vic.). After further processing as described below, the cells were suspended in a culture medium (Medium 199; C.S.L., Melbourne, Vic., with 20–40 per cent guinea-pig serum).

Heat-killed lymphocytes were prepared by incubating 1 ml suspension containing 0·6–·8 × 10^6 lymphocytes in Hanks's solution at 56° for 30 minutes. Cell death was ascertained ·y the eosin technique, i.e. by incubating the cells for 5–10 minutes in 0·5 per cent solution ·f Eosin B (Matheson, Coleman and Bell, New Jersey, U.S.A.) in saline, and counting a ·otal of 200–300 eosin-stained and unstained cells from each preparation (Hanks and Wallace, 1958).

Preparation of peritoneal cells

Four guinea-pigs were injected intraperitoneally with 20 ml Hanks's solution and then killed. The peritoneal cavities were opened and the fluid was aspirated and centrifuged at 200 ***g*** for 10 minutes. The pellet of cells from each animal was washed twice with Hanks's solution and then suspended in culture medium.

Preparation of blood macrophages

Cardiac blood from four guinea-pigs was sedimented with 'Methocel' as described above. The upper two-thirds of the leucocyte-enriched layer were then discarded, while the lower third was centrifuged at 200 ***g*** for 10 minutes. The cells in the resulting pellet were washed twice with Hanks's solution and suspended in culture medium.

Cell counts

Total white cell counts were done in Neubauer counting chambers using 2 per cent aqueous acetic acid as diluent. Differential counts were performed on smears prepared from pellets obtained by centrifuging the cell suspension and stained with May–Grünwald-Giemsa.

The percentage of transformed cells was determined by counting at least 300 cells from each preparation using the longitudinal method of Dacie and Lewis (1963).

Conjugation with DNFB

(a) *Bovine serum albumin* (BSA). Two cuvettes (in the same light path) were placed in the reference and two in the test compartment of a Unicam S.P. 800 B spectrophotometer. One cuvette in each compartment contained BSA (20 mg/ml) and the other DNFB (20 μg/ml), both in buffer at the appropriate pH. After equilibration for 5 minutes the solutions in the test compartment were mixed to give an effective concentration of 10 mg/ml and 10 μg/ml for BSA and DNFB, respectively; the cuvettes were then replaced. The reaction was followed for 72 hours at 325 mμ, since it had been previously found that at this wavelength there was a maximal difference between the absorbance of BSA and DNFB and the DNFB–BSA conjugate. The pH of the reaction mixture was then lowered to about 3 with 2N hydrochloric acid. An equivalent volume of distilled water was added to the reference cells to obtain a corresponding dilution of DNFB and BSA. The optical density of the reaction mixture was then again determined. This procedure was adopted to measure any absorbance due to a coloured compound (presumably 2,4-dinitrophenol; DNP) which slowly formed on standing in alkaline DNFB solutions.

(b) *Lymphocytes*. In preliminary experiments 10^6 twice washed leucocytes were incubated with 0–100 μg/ml concentrations of DNFB in phosphate buffer pH 8·5 for periods up to 10 hours. In subsequent experiments the lymphocyte pellet was reconstituted in a solution of DNFB (10 μg/ml) in the phosphate buffer. The volume of the DNFB solution was adjusted to give a final concentration of about 10^6 cells/ml. After 30 minutes incubation, the

suspension was reduced to pH approximately 6·3 by gassing with carbon dioxide, centrifuged at 200 *g* for 5 minutes, the supernatant removed, the cells washed thrice with Hanks's solution and then suspended in culture medium. Control cultures of unconjugated lymphocytes were treated in a similar fashion, except that the cells were not incubated with DNFB.

(c) *Erythrocytes.* Erythrocytes obtained from the residual defibrinated and sedimented blood by centrifugation for 10 minutes at 200 *g* were washed twice with Hanks's solution and conjugated with DNFB as described above for lymphocytes.

(d) *Serum proteins.* Autologous serum proteins were conjugated with DNFB by mixing equal portions of serum (obtained from centrifuged blood) with DNFB solution (containing 10 μg DNFB per ml) and incubating the mixture at 37° for 30 minutes.

Preparation of mixed cultures

Unconjugated live lymphocytes ($0{\cdot}6$–$1{\cdot}3\times10^6$) were mixed with either an approximately equal number of autologous: (1) conjugated heat-killed lymphocytes, (2) unconjugated heat-killed lymphocytes, (3) conjugated erythrocytes, or with 2 ml autologous conjugated serum protein solution. A fifth set of mixed cultures was prepared by mixing $0{\cdot}6$–$0{\cdot}8\times10^6$ conjugated live lymphocytes with a similar number of autologous unconjugated heat-killed lymphocytes.

Carbon studies

A carbon suspension was prepared by diluting 'Pelikan' ink (Batch No. C11/1431a Gunther Wagner Werke, West Germany) in proportion 1 : 5 with Hanks's solution. Approximately 0·05 ml of the carbon suspension was added to: (1), 3 ml of each peritoneal cell preparation (10^6 cells/ml); (2), 1 ml of each blood macrophage preparation (10^6 leucocytes/ml); (3), 1 ml of each lymphocyte preparation ($0{\cdot}5\times10^6$ lymphocytes/ml). The cells were incubated with carbon at 37° for 2 hours, then centrifuged and washed 3 or 4 times with Hanks's solution. After the last centrifugation the cells were spread on microscope slides and stained with May-Grünwald-Giemsa.

Uptake of tritiated thymidine

Thymidine-6-T(n) (Radiochemical Centre, England) was used in amounts of 2 μCi per 10^6 lymphocytes. In the first experiment duplicate cultures of conjugated live lymphocytes from two sensitized and two unsensitized guinea-pigs were incubated with thymidine for 5 days. Each day the cultures were sampled and the uptake of thymidine was determined. In the second experiment portions of 3-day duplicate cultures of conjugated lymphocytes from two sensitized guinea-pigs were pulsed with thymidine for 1, 2, 4 or 6 hours.

In the third experiment duplicate cultures of conjugated lymphocytes were prepared from four sensitized guinea-pigs. Portions of 3-day cultures were pulsed with thymidine for 2 hours. The remainder of each culture was incubated for a further 2 days and the percentage of transformed cells then determined. In the fourth experiment duplicate cultures were prepared of both conjugated and unconjugated lymphocytes from twelve sensitized and twelve unsensitized guinea-pigs. From a further seven sensitized animals were established: (1), seven mixed cultures of unconjugated live lymphocytes and autologous conjugated erythrocytes; (2), five cultures of conjugated live lymphocytes; (3), five mixed cultures of unconjugated live lymphocytes and autologous conjugated serum proteins; (4), four mixed cultures of unconjugated live lymphocytes and autologous conjugated

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Autoradiography

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CONJUGATION OF 1-F

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FIG. 1. The effect
albumin.

heat-killed lymphocytes. All cultures were pulsed with thymidine for 2 hours on the 3rd day of incubation. After incubation for the stated periods, the cells were washed thrice with Hanks's solution and then prepared for liquid scintillation counting (Tri-Carb, Packard) according to the method of Mahin and Lofberg (1967).

Autoradiography

Cultures of conjugated lymphocytes were prepared from the cardiac blood of four sensitized guinea-pigs and unconjugated lymphocytes from the blood of two unsensitized animals. Subsequently, $0{\cdot}5\times10^6$ unconjugated lymphocytes were incubated with 2 μCi tritiated thymidine for 16 hours. Portions containing approximately $0{\cdot}5\times10^6$ conjugated lymphocytes from cultures aged 0, 3 or 5 days were also incubated with 2 μCi tritiated thymidine for 16 hours. After incubation the cells were washed thrice with Hanks's solution, centrifuged and spread on microscope slides. The slides were coated with NTB2 autoradiographic emulsion (Eastman-Kodak, U.S.A.), exposed for 5 days, developed and then stained with May-Grünwald-Giemsa.

RESULTS

CONJUGATION OF 1-FLUORO-2,4-DINITROBENZENE (DNFB) WITH BOVINE SERUM ALBUMIN (BSA)

The conjugation of DNFB with protein is known to occur rapidly at alkaline pH values (Eisen, Orris and Belman, 1952). The extent of conjugation of DNFB with protein in the range suitable for use with living cells *in vitro* was ascertained using BSA. BSA was chosen for convenience, because light scattering of cell suspensions rendered them unsuitable for direct spectrophotometric measurement of DNFB conjugation. The reaction rates of BSA with DNFB at different pH values are illustrated in Fig. 1. It is noteworthy that appreciable conjugation occurs even at pH 5·5.

During this investigation a yellow colour, probably due to 2,4-dinitrophenol, was found to develop gradually in alkaline DNFB solutions standing for several weeks. This colour

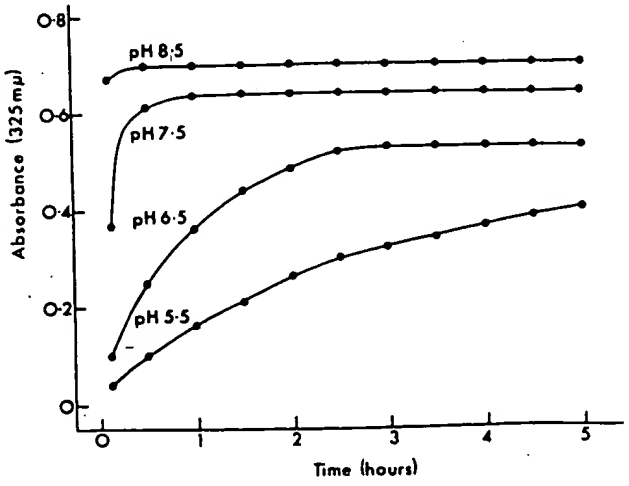

FIG. 1. The effect of pH on the rate of conjugation of 1-fluoro-2,4-dinitrobenzene with bovine serum albumin.

disappeared at about pH 3, unlike that of DNFB–albumin conjugates. Accordingly, the absorbance of the reaction mixtures was measured after 72 hours both at the reaction pH (see Fig. 1) and at pH 3. After correction for dilution by acid, absorbance was similar at both pH values, suggesting that the formation of the yellow colour was not sufficiently rapid to interfere appreciably with the measurements recorded in Fig. 1. In view of the results with BSA (Fig. 1) conjugation of DNFB with living cells was carried out at pH 8·5 for 30 minutes.

CONJUGATION OF DNFB WITH LEUCOCYTES

Preliminary experiments showed that preparations of 10 μg DNFB per 10^6 leucocytes contained no more eosin-stained (non-viable) leucocytes than control cultures containing no DNFB. A concentration of 10 μg DNFB per 10^6 cells was therefore selected for subsequent conjugation. A preparation of 10^6 leucocytes and the same number of erythrocytes contained approximately 3·3 mg protein.

DIFFERENTIAL COUNT

Differential counts of leucocyte suspensions immediately prior to culture showed that virtually all (97–100 per cent) cells were lymphocytes, and the remainder granulocytes and occasional monocytes. The suspensions contained approximately equal numbers of red and white cells.

TOTAL CELL COUNT

The total cell count decreased by up to 30 per cent by the 3rd day of culture but usually diminished no further in the subsequent 2 days (Table 1). Two possible exceptions were the counts of conjugated lymphocytes and lymphocytes incubated with conjugated erythrocytes (both from sensitized donors) which, on the 5th day, fell to about 50 per cent of

TABLE 1

EFFECT OF INCUBATION ON CELL COUNTS IN CULTURE

	No. of animals	Serum	No. of cells per ml ($\times 10^6$) 0 days	3 days	5 days
Conjugated lymphocytes					
unsensitized donors	14§	A	1·2–2·0 (1·6)	1·1–1·4 (1·3)	1·1–1·5 (1·3)
	2	H*	1·9 (1·9)	1·6, 1·7 (1·65)	1·6 (1·6)
sensitized donors	25‡§	A	0·8–2·0 (1·3)	0·7–1·4 (0·9)	0·3–1·1 (0·4)
	2	H†	1·8, 2·0 (1·9)	1·6, 1·7 (1·65)	1·7, 1·8 (1·75)
Conjugated red cells sensitized donors	17‡§	A	0·8–1·3 (0·9)	0·8–1·1 (0·9)	0·4–0·6 (0·5)
Conjugated serum proteins sensitized donors	17‡§	A	0·8–1·3 (1·0)	0·6–1·2 (0·9)	0·7–0·9 (0·8)
Unconjugated lymphocytes					
unsensitized donors	2	H†	2·0 (2·0)	1·5, 1·7 (1·6)	1·4, 1·7 (1·55)
	4	A	1·1–2·0 (1·5)	0·9–1·6 (1·2)	1·3–1·8 (1·5)
sensitized donors	14	A	1·1–1·8 (1·3)	1·0–1·5 (0·9)	0·9–1·4 (1·1)
	4	H*	1·2–1·8 (1·5)	1·0–1·6 (1·2)	1·2–1·4 (1·3)

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

their values on the 3rd day (Table 1). This relatively pronounced decrease in the number of lymphocytes possibly indicates *in vitro* lysis of sensitive cells exposed to DNFB or to dinitrophenylated erythrocytes. A similar decrease was not observed in lymphocyte cultures from sensitized donors incubated with dinitrophenylated serum proteins. In mixed cultures of autologous live and heat-killed lymphocytes, either of which was conjugated with DNFB, the total cells decreased by the 5th day to about one-third the initial number. This decrease corresponded to that observed in cultures of only live lymphocytes (Table 1).

VIABLE CELL COUNT

On the day of collection (0 day) 77–100 per cent of all cells were viable; on the 3rd and 5th day of culture, the viability decreased to 30–80 per cent and 10–75 per cent respectively. The percentage of viable cells varied widely from culture to culture (Table 2) and no special trends of either decreased or increased viability could be discerned. In mixed cultures of live and autologous heat-killed lymphocytes, the decrease in the percentage of viable cells (from 40 to 60 per cent initially to 20–40 per cent on the 3rd or 5th day) was proportional to that in cultures containing only live lymphocytes.

TABLE 2

EFFECT OF CULTURE ON VIABLE CELL COUNT

	No. of animals	Serum	Per cent viable cells		
			0 days	3 days	5 days
Conjugated lymphocytes unsensitized donors	14§	A	95–100 (96)	58–71 (61)	39–58 (43)
	2	H*	96, 97 (96·5)	52, 60 (56)	18, 41 (30)
sensitized donors	25‡§	A	85–100 (92)	36–80 (60)	10–58 (41)
	2	H†	95, 100 (97·5)	52, 58 (55)	19, 24 (27)
Conjugated red cells sensitized donors	17‡§	A	79–100 (91)	33–80 (71)	28–60 (53)
Conjugated serum proteins sensitized donors	17‡§	A	77–100 (93)	32–70 (59)	26–60 (47)
Unconjugated lymphocytes unsensitized donors	4	A	95–100 (97)	58–80 (67)	20–75 (50)
	2	H†	95, 100 (98)	60 (60)	31, 48 (40)
sensitized donors	14	A	91–100 (93)	58–65 (60)	38–50 (43)
	4	H*	92–98 (96)	45–60 (52)	30–68 (40)

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

LYMPHOCYTE TRANSFORMATION

Only a small number (<2 per cent) of transformed cells was seen in mixed cultures of unconjugated live lymphocytes and either autologous conjugated serum proteins (in two of seventeen cultures) or autologous conjugated erythrocytes (in six of seventeen cultures). By contrast, 8–22 per cent transformed cells (Fig. 12) was seen in 5-day cultures of live lymphocytes conjugated with DNFB (Table 3). Similarly, 5–10 per cent transformed cells were present in 5-day mixed cultures of conjugated live lymphocytes and autologous unconjugated heat-killed lymphocytes; the proportion of transformed cells would have been even higher (10–20 per cent) if allowance had been made for the presence of about 50 per cent non-viable lymphocytes in the initial inoculum.

TABLE 3

EFFECT OF CONJUGATION ON TRANSPORMATION OF CULTURED LYMPHOCYTES

	No. of animals	Serum	Per cent transformed cells	
			3 days	5 days
Conjugated lymphocytes				
unsensitized donors	14§	A	0	0
	2	H*	0	0
sensitized donors	25‡§	A	0	8–22 (14)
	2	H†	0	8, 16 (12)
Conjugated red cells				
sensitized donors	17‡§	A	0	0–1 (<1)
Conjugated serum proteins				
sensitized donors	17‡§	A	0	0–2 (<1)
Unconjugated lymphocytes				
unsensitized donors	4	A	0	0
	2	H†	0	0
sensitized donors	14	A	0	0
	4	H*	0	0

A: autologous. H: homoglogous.
* Serum from sensitized donors. † Serum from unsensitized donors. ‡ Includes the eleven guinea-pigs whose lymphocytes were also incubated with dinitrophenylated red cells and serum proteins. § Cultures from these animals were prepared in duplicate. Values in parentheses represent means.

CARBON UPTAKE

Carbon-laden cells were observed both in preparations of blood macrophages and peritoneal cell cultures aged 0, 3 or 5 days. By contrast, no cells which phagocytosed carbon were detected in 0-, 3- or 5-day cultures of conjugated live lymphocytes from sensitized donors.

UPTAKE OF TRITIATED THYMIDINE

In an initial experiment conjugated lymphocytes from two unsensitized guinea-pigs incorporated relatively little radioactivity (<2500 counts/min per 10^6 lymphocytes) in the first 4 days of incubation with tritiated thymidine but took up rather more radioactivity on the 5th day (about 18,000 counts/min per 10^6 lymphocytes). By contrast, conjugated lymphocytes from two sensitized animals incorporated respectively about 25,000 counts min and 55,000 counts/min per 10^6 lymphocytes on the 3rd and 5th day of incubation.

TABLE 4

LACK OF CORRELATION BETWEEN UPTAKE OF TRITIATED THYMIDINE AND TRANSFORMATION IN CULTURES OF CONJUGATED LYMPHOYCTES

	Sensitized donors							
	1		2		3		4	
	a	b	a	b	a	b	a	b
Radioactivity in 3-day cultures (counts/min/10^3 cells)	135	132	135	134	83	64	90	74
Transformed cells in 5-day cultures (per cent)	16	9	11	12	10	9	10	7

a, b: duplicate cultures.

TABLE 5

EFFECT OF DONOR SENSITIVITY ON UPTAKE OF TRITIATED THYMIDINE BY CULTURED LYMPHOCYTES

	No. of animals	Radioactivity (counts/min per 10^6 live lymphocytes)
ensitized donors		
Conjugated red cells	7[a]	110–880 (480)
Conjugated serum proteins	5[a]	96–700 (360)
Conjugated killed lymphocytes	4[a]	580–830 (696)
Conjugated live lymphocytes	17[a,b]	15,000–132,500 (73,000)
Unconjugated live lymphocytes	12[b]	38–300 (73)
Unsensitized donors		
Conjugated live lymphocytes	12[c]	38–500 (88)
Unconjugated live lymphocytes	12[c]	34–550 (110)

The superscripts a, b and c designate different batches of animals.

In the second experiment 3-day cultures of conjugated lymphocytes from two sensitized guinea-pigs incorporated 20–30 times more thymidine than similar cultures of unconjugated lymphocytes. Furthermore, conjugated lymphocytes pulsed with thymidine for 2 hours took up almost as much radioactivity (29,000 counts/min per 10^6 lymphocytes) as lymphocytes pulsed for 6 hours (32,000 counts/min per 10^6 lymphocytes).

In the third experiment, duplicate cultures of conjugated lymphocytes from four sensitized animals exhibited an increased uptake of thymidine on the 3rd day of incubation and the presence of transformed cells on the 5th day. However, the uptake of radioactivity appeared not to vary with the percentage of transformed cells (Table 4).

The results of the fourth experiment have been recorded in Table 5. Tritiated thymidine was incorporated in relatively low amounts (<880 counts/min per 10^6 lymphocytes) in cultures of: (1) conjugated lymphocytes from unsensitized donors, (2) unconjugated lymphocytes from either sensitized or unsensitized donors, (3) mixed cultures of unconjugated live lymphocytes from sensitized donors and autologous conjugated (a) erythrocytes, (b) serum proteins, or (c) heat-killed lymphocytes. By contrast, the uptake of radioactivity exceeded 15,000 counts/min per 10^6 lymphocytes in all cultures of conjugated live lymphocytes from sensitized guinea-pigs.

AUTORADIOGRAPHY

Preparations of freshly collected unconjugated lymphocytes from unsensitized guinea-pigs contained no cells which became labelled after 16 hours incubation with tritiated thymidine. By contrast, in 16-hour cultures of conjugated lymphocytes from sensitized donors, 6 (0·34 per cent) of 1750 examined leucocytes were labelled. The labelled cells resembled large lymphocytes (Figs. 2–4, cf. Fig. 5). In 3-day cultures, 48 (6·9 per cent) of 700 examined leucocytes were labelled. The labelled cells varied from ones resembling large lymphocytes to others which approached the appearance of transformed cells (Figs. 6-8, cf. Fig. 9). They also varied in the degree of labelling (Figs. 6–9). Two cells which exhibited markedly different labelling were united by a cytoplasmic strand (Fig. 10), probably depicting the last stage in cellular division. In 5-day cultures, 120 (18·2 per cent) of 660 examined leucocytes were labelled. However, only 58 (8·8 per cent) of these were 'typical' transformed cells (Fig. 11, cf. Fig. 12), the other 62 (9·4 per cent) appearing to represent intermediate stages between a large lymphocyte and a transformed cell. The

FIG. 3.

FIG. 2.

FIG. 5.

FIG. 4.

B

F. 202-5. Ly cox/dw/DNFB+ incub'd w/tritiated thymidine for 16 hrs — the cells exhibit scanty cytoplasm + variable degree of labelling; unlabelled [illegible] in Fig. 5. ×220).

FIG. 6.

FIG. 7.

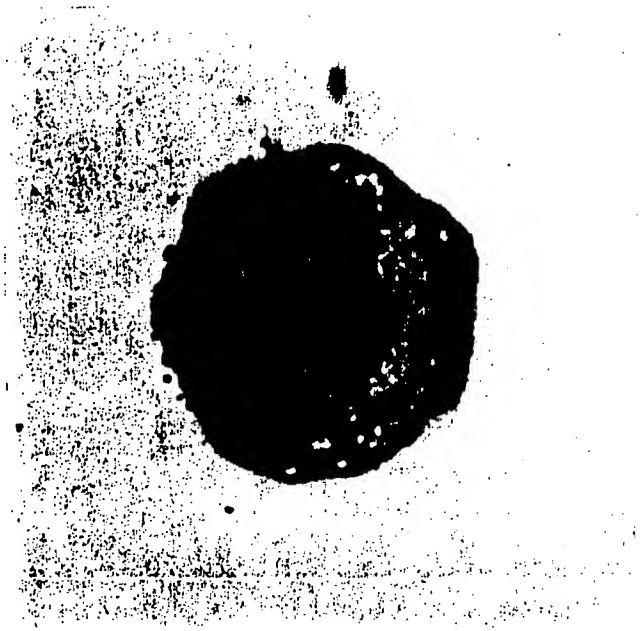

FIG. 8.

FIG. 9.

FIGS. 6–9. Cells from a 3-day-old culture of dinitrophenylated lymphocytes, illustrating variation in size and degree of labelling after 16 hours incubation with tritiated thymidine; an unlabelled cell from the same culture is shown in Fig. 9. ×2200.

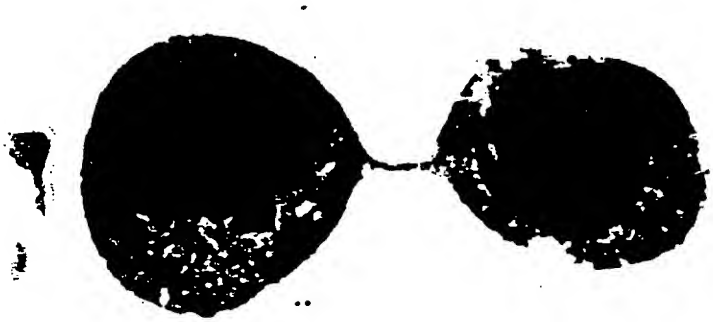

FIG. 10. Cells from a 3-day-old culture of dinitrophenylated lymphocytes. The cells are joined by a strand of cytoplasm. ×2200.

FIG. 11. A labelled, transformed cell from a culture aged 5 days. ×2200.

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FIG. 12. A transformed cell from a culture aged 5 days, stained with May-Grünwald-Giemsa. ×2200.

degree of labelling varied considerably in different cells but did not seem to be related to the microscopic appearance of the cells. Nevertheless, the majority of transformed cells (62·4 per cent) were labelled.

DISCUSSION

The transformation of lymphocytes *in vitro* in the presence of specific antigens has been amply documented (Pearmain, Lycette and Fitzgerald, 1963; Elves, Roath and Israëls, 1963; Coulsen and Chalmers, 1967). However, despite the frequent use of DNCB in investigations of delayed hypersensitivity (Landsteiner and Jacobs, 1935; Turk, 1967; Bloom and Chase, 1967), no studies appear to have been made of its effect on cultured lymphocytes. The use of DNFB in this work was prompted by its ability to form stable conjugates with protein (Eisen, Orris and Belman, 1952).

In defining the conditions for the use of DNFB the extent of its conjugation with BSA has been investigated in the pH range similar to which lymphocytes can be exposed.

It may be assumed that after 4 hours at pH 8·5, no further reaction occurs between DNFB and BSA since there is virtually no additional increase in optical density after 30 minutes. Therefore, it seems reasonable to assume that the reaction between DNFB and protein in the cell suspensions is also approximately complete in 30 minutes. However, the availability of cell protein for conjugation may be limited, on one hand, by the adherence of serum protein to the cells (despite washing), and, on the other hand, by the intracellular localization of the bulk of the protein. It seems unlikely that free DNFB can enter the cells in view of its high reactivity at the usually alkaline pH of the extracellular fluid, and the availability of protein for conjugation in both the adsorbed serum

and the cell membrane. However, if DNFB does enter the cell, it can presumably react with intracellular protein since some conjugation with albumin is found even at pH 5·5.

In cultures of conjugated cells from the sensitized guinea-pigs, the total number of cells decreases by the 5th day to about one-third of its initial value. Since a similar fall does not usually occur in other cultures, the conjugated cells from sensitized donors probably undergo lysis of immune origin. Moreover, since the total number of cells similarly decreases in cultures of unconjugated heat-killed cells used with conjugated live cells, as well as unconjugated live cells used with conjugated heat-killed cells, dinitrophenylated lymphocyte antigen can possibly induce lysis of unconjugated living lymphocytes. By contrast, it appears that transformation can be induced only in cells directly exposed to DNFB.

The transformation of conjugated lymphocytes from sensitized donors has been established both on a morphological basis and by the incorporation of tritiated thymidine. It appears to be a specific reaction of conjugated, sensitive lymphocytes, since neither transformed cells nor appreciable thymidine uptake are observed in unconjugated cell cultures from sensitized animals or conjugated and unconjugated cell cultures from unsensitized donors. The transformation appears to be a specific outcome of the exposure of sensitized lymphocytes to DNFB, since little or no transformation occurs when live lymphocytes are incubated with either conjugated, heat-killed lymphocytes, erythrocytes or serum proteins.

In some cultures containing conjugated erythrocytes or serum proteins, the presence of a small number of transformed cells can be interpreted as a low degree of responsiveness to these antigens. It is also possible, however, that a small amount of residual unconjugated DNFB remains adsorbed to erythrocytes or is present in serum and later reacts with lymphocytes.

Conjugation of DNFB with lymphocytes therefore appears to be a necessary prerequisite to transformation. The high reactivity of DNFB suggests that it mainly combines with protein adsorbed to the cell or a component of the cell membrane. Since the adsorbed protein is probably derived from the serum, and conjugated serum proteins do not induce appreciable transformation, it seems likely that the union resulting in transformation occurs at the cell membrane. Transformation of lymphocytes from sensitized animals following *in vitro* conjugation with DNFB suggests that a similar mechanism may operate *in vivo*.

Migrating lymphocytes may initially react with the chemical in the skin and induce hypersensitivity after undergoing changes either locally or in the regional lymph nodes. Sensitive lymphocytes may subsequently react with freshly applied chemical and having been stimulated by the specific antigen, release factors which elicit the local reaction.

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Exhibit 12

Applicant ✓

Clinical Investigation

Clinical Responses With Active Specific Intralymphatic Immunotherapy for Cancer—A Phase I-II Trial

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We evaluated the method of active specific intralymphatic immunization to treat cancer in 32 patients with various tumor types as part of a broad-based phase I-II evaluation and describe the results of 3 sequential series. In series 1, the patients (n = 13) received 2 or more injections of autologous, cryopreserved, irradiated tumor cells directly into the lymphatic system through the cannulation of a dorsal pedal lymphatic channel. In series 2, the patients (n = 7) received low-dose cyclophosphamide, 300 mg per m², 3 days before the autologous cell vaccine was administered. Series 3 (12 patients) was similar to series 2 except that the tumor cells were treated with cholesteryl hemisuccinate immediately before irradiation. Patients received from 2 to 6 injections of cells, depending on availability, at 2-week intervals. In all, 91 treatments are evaluated in this study. Clinical responses occurred in 7 of the 32 patients and were seen in all 3 series with about the same frequency. These responses occurred in cases of melanoma, lung cancer, colon cancer, and sarcoma. Regressions occurred in both visceral and subcutaneous sites. There was little toxicity, the chief side effect being local discomfort or inflammation. This experience indicates that active specific intralymphatic immunotherapy is safe, produces antitumor effects, and requires more investigation to increase the frequency and duration of observable tumor regression.

(Wiseman CL, Rao VS, Kennedy PS, et al: Clinical responses with active specific intralymphatic immunotherapy for cancer—A phase I-II trial. West J Med 1989 Sep; 151:283-288)

The historical enthusiasm for using autologous tumor cells as immunogens has resulted in a number of clinical trials, and some positive results warrant further attention.[1] We have been interested in several recent developments that encourage further investigation of such an approach. We began a study to investigate and extend the original report of Juillard and co-workers, who described regression of metastatic tumors after inoculating irradiated tumor cells directly into the lymph nodes through the dorsal pedal lymphatic vessels.[2]

Direct intralymphatic immunization is an attractive concept for a number of theoretic reasons. Such a technique could circumvent possible immunosuppressive mechanisms of a primary tumor. Regional lymph nodes vary widely in immunologic responsiveness.[3] Some studies indicate that nodes close to a primary tumor have decreased functional competence and increased suppressor-cell activity as compared with nodes distant from the primary tumor.[4]

The intralymphatic approach has been studied in animals[5,6] with positive results and in several human tumor categories. In addition to the work of Juillard and colleagues, augmented immunologic responses were reported in breast cancer and in renal cancer by Adler and associates, although the clinical significance of these responses remains open.[7,8]

Our initial findings suggested that the method reproducibly elicited substantial biologic effects, with a significant elevation of the fraction (and absolute number) of circulating CD4+ lymphocytes. We observed that this elevation occurred after each immunization and in almost every patient.[9,10] In a few melanoma patients, two subpopulations of CD4+ were identified.[11]

Since the initiation of this study, we have made several modifications based on possibly useful newer techniques. The reports by Berd and Mastrangelo indicated that the use of low doses of cyclophosphamide may selectively block suppressor-cell functional activity, thereby leading to augmented specific immune responses.[12] After our initial experience, we introduced this as a pretreatment for our patients (series 2 and 3). We then changed the program in an attempt to increase potential cell-surface immunogenicity of tumor cells by reducing membrane-lipid microviscosity. We introduced the methods of Skornick and associates, using a brief incubation with cholesteryl hemisuccinate (series 3).[13]

Patients and Methods

Tumor Vaccine

Surgically removed tumor was dispersed by collagenase and deoxyribonuclease according to methods previously de-

From the Wilshire Oncology Medical Group (except Dr Rao), the Los Angeles Oncologic Institute, and the St Vincent Medical Center, Los Angeles.

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scribed.[9] The cells were frozen slowly using a programmed cryogenic freezer (Union Carbide, Indianapolis) and stored over liquid nitrogen. On demand, the cells were thawed rapidly and washed in Hanks' medium. Adherent cells were removed by incubating at 37°C, and the remaining population was then irradiated to 200 gray from a cobalt 57 source. Dosimetry and supervision of the radiation procedure were provided by Armand Bouzaglou, MD, and John Sevilla, MD. For patients in series 3, vaccine preparation was modified. After thawing and washing, the cells were suspended in a polyvinyl pyrolidone-Hanks' solution, pH 7.2, containing 250 μg per ml cholesteryl hemisuccinate (Sigma), adjusted to 1×10^6 viable cells per milliliter, and incubated three to four hours at room temperature, washed, and resuspended in lactated Ringer's solution 5 to 10×10^6 viable cells per milliliter before irradiation. The irradiated cells were suspended in a lactated Ringer's solution at a concentration of 10 to 15 $\times 10^6$ viable cells per milliliter. Viability was assessed by trypan blue exclusion. Aliquots of the initial preparation and the irradiated vaccine were routinely tested for bacterial contamination by culture as recommended by federal protocol.[14] The viability of the tumor cells was generally about 70% or higher, although occasionally a patient was treated with a vaccine of lower viability.

Patients

All patients provided signed informed consent before enrolling in this program. Approval and periodic review had been provided by the Institutional Review Board of the St Vincent Medical Center (Los Angeles), both initially and after the sequential modifications of the program. All patients received a complete medical history and physical examination, complete blood counts, biochemical profile, and such x-ray films, computed tomograms, and isotope scans as needed to evaluate the extent of metastatic involvement and the dimensions of at least one measurable indicator lesion. Therapy was not initiated until at least three weeks had elapsed from previous chemotherapy, radiation therapy, or surgical procedure requiring general anesthesia. No additional antitumor therapy was permitted for any patient during the period of treatment on this protocol.

Autologous irradiated tumor cells, suspended at a concentration of 10 to 15×10^6 viable cells per milliliter, were injected over several minutes into a dorsal pedal lymphatic

TABLE 1.—*Clinical Characteristics of 32 Patients Receiving Active Specific Intralymphatic Immunotherapy*

Patient	Age, yr	Sex	Primary Cancer	ECOG Scale of Metastases*	Site(s) of Metastases	Previous Chemotherapy Regimens, No.
Series 1						
1	58	♂	Melanoma	1	Lung	0
2	45	♂	Melanoma	1	Lung, liver	0
3	76	♂	Melanoma	1	Nodes	3
4	28	♂	Melanoma	4	Skin	3
5	74	♂	Melanoma	4	Lung, liver	0
6	44	♂	Colon	4	Nodes, ascites, liver	1
7	28	♂	Colon	2	Liver	0
8	75	♀	Colon	2	Liver	1
9	54	♂	Lung	2	Lung	0
10	35	♂	Lung	1	Lung	0
11	41	♂	Nasopharynx	4	Liver, bone, lung	3
12	64	♂	Renal	1	Lung, chest wall	0
13	61	♂	Renal	2	Kidney	0
Series 2						
14	61	♂	Colon	2	Lung, abdomen	1
15	46	♂	Colon	1	Liver	0
16	32	♂	Melanoma	3	Liver, lung	0
17	46	♀	Melanoma	1	Skin	2
18	42	♀	Melanoma	2	Abdomen, nodes	1
19	48	♂	Renal	2	Lung	0
20	41	♂	Unknown	3	Abdomen, lung	1
Series 3						
21	64	♀	Colon	2	Liver, bone	1
22	52	♀	Lung	4	Skin, lung, kidney	1
23	76	♂	Lung	3	Lung, liver	0
24	68	♂	Melanoma	2	Abdomen	1
25	45	♂	Melanoma	2	Abdomen, nodes	3
26	56	♂	Melanoma	3	Scalp, liver, nodes	3
27	36	♀	Melanoma	2	Lung, nodes	0
28	32	♂	Melanoma	1	Lung	3
29	62	♂	Melanoma	1	Lung, skin	1
30	45	♀	Melanoma	3	Lung	2
31	65	♂	Renal	4	Bone, lung	3
32	45	♀	Sarcoma	2	Skin, liver	1

ECOG—Eastern Cooperative Oncology Group

*The ECOG scale measures the performance status of a patient using a scale of 0 (fully active, able to carry on all predisease activities without restrictions) to 4 (completely disabled, totally confined to bed or chair).

channel isolated after Evans blue was infiltrated and lidocaine hydrochloride was administered for local anesthesia according to methods used for lymphangiography. All cutdown procedures were done by the staff of the Department of Radiology, St Vincent Medical Center. Patients received the vaccine at two- to four-week intervals as supplies permitted. Unless required for other medical considerations, admission to hospital was not needed. For those patients receiving cyclophosphamide (Cytoxan, Bristol-Myers Oncology Division), the drug was injected intravenously three days before vaccine inoculation at a dose of 300 mg per m^2 following pretreatment with the intramuscular administration of triethylperazine, 10 to 15 mg (Torecan, Roxane Laboratories, Inc).

TABLE 2.—*Distribution of Tumor Types Per Series*

Tumor Types	Patients, No.			
	Series 1	Series 2	Series 3	Total
Melanoma	5	3	7	15
Colon cancer	3	2	1	6
Lung cancer	2	0	2	4
Renal cancer	2	1	1	4
Miscellaneous*	1	1	1	3
Total	13	7	12	32

*Nasopharyngeal 1, adenocarcinoma of unknown primary 1, atrial myxosarcoma 1.

Results

The roster of patients, together with clinical characteristics, is provided in Table 1. As mentioned, our initial experience, series 1, represents the tumor vaccine program unmodified. Series 2 and 3 differ in that patients were treated with low-dose cyclophosphamide. Series 3 comprises those patients whose vaccine underwent pretreatment with cholesteryl hemisuccinate; additionally, series 3 patients, similar to the patients in series 2, received a priming, immunomodulating dose of cyclophosphamide before the vaccine treatment. There were 13 patients in series 1, 7 in series 2, and 12 in series 3. The program was initiated June 1, 1981; we evaluated responses and survival as of July 15, 1988. Table 2 shows the tumor types and their distribution according to series.

All patients had advanced cancer, usually with pulmonary or intra-abdominal metastases. The median age was 48 years (range, 26 to 78). There were 8 women. Eleven patients had had no previous therapy; an occasional patient had had extensive previous treatment. The median performance score on the Eastern Cooperative Oncology Group scale was 2; six had scores of 4 (completely bedridden) and five had

TABLE 3.—*Therapy and Response*

Patient	Primary Cancer	Viable Cells/Cycle, × 10^8/ml	Tumor Response	Time to Progression, wk	Survival, wk	Comment
Series 1						
1	Melanoma	5.0, 25.0, 50.0, 56.0	Mixed	12.0	46.1	Partial remission, pulmonary; CNS failure
2	Melanoma	20.0, 15.0	Progression	4.0	13.0	
3	Melanoma	10.0, 14.0, 20.0	Progression	10.0	146.7	
4	Melanoma	20.0, 40.0	Progression	9.1	9.1	
5	Melanoma	10.0, 5.0, 35.0, 60.0, 20.0	Progression	12.9	13.1	
6	Colon	3.0, 6.0	Mixed	4.0	7.7	Regression of Virchow's node
7	Colon	6.0, 4.5, 4.8	Stable	19.7	24.0	
8	Colon	6.0, 9.0, 33.0	Stable	32.4	43.3	
9	Lung	10.0, 20.0, 12.0	Progression	9.0	19.1	
10	Lung	10.0, 2.0	Complete remission	22.7	43.1	See text
11	Nasopharynx	14.0, 8.0, 1.5	Progression	7.7	14.4	
12	Renal	20.0, 10.0, 30.0	Progression	10.0	209.4	
13	Renal	14.0, 14.0, 17.0	Progression	6.0	6.0	
Series 2						
14	Colon	10.0, 12.0, 10.0	Mixed	14.3	73.9	See text
15	Colon	11.0, 14.0, 14.0	Progression	8.3	169.4	Active, slowly progressive disease
16	Melanoma	20.0, 18.0	Progression	3.6	37.7	
17	Melanoma	16.0, 21.0, 12.5	Complete remission	57.9	79.0	See text
18	Melanoma	24.0, 28.0, 30.0	Stable	13.4	21.9	
19	Renal	12.0, 12.0, 13.0, 8.0, 7.0, 6.0	Progression	6.9	45.7	
20	Unknown	6.0, 9.0	Progression	7.0	9.9	
Series 3						
21	Colon	11.0, 6.0	Progression	7.0	15.6	
22	Lung	10.0, 9.7	Progression	4.6	6.1	
23	Lung	9.0, 9.0	Progression	7.0	19.3	
24	Melanoma	13.0, 9.0, 9.0	Progression	19.0	57.7	
25	Melanoma	10.0, 10.0, 10.0	Progression	10.0	59.3	
26	Melanoma	10.0, 12.0	Progression	5.7	6.4	
27	Melanoma	6.0, 8.0	Progression	12.4	17.1	
28	Melanoma	13.0, 13.0, 12.0, 10.0	Progression	9.0	39.7	
29	Melanoma	10.0, 10.0, 11.0	Partial remission	25.1	59.7	
30	Melanoma	8.0, 9.0, 9.0	Progression	6.9	13.1	
31	Renal	17.0, 18.0	Progression	4.4	11.9	
32	Sarcoma	11.0, 12.0, 14.0	Mixed	5.3	42.9	See text

CNS—central nervous system

scores of 3—that is, about a third of the patients were partially or completely bedridden.

Table 3 depicts the number of cycles of immunization per patient, the amount of viable cells of each vaccine treatment, the maximum response according to standard criteria of the Southwestern Oncology Group, the time to progression, and the duration of survival from the initiation of treatment. For 32 patients in the study, there were a total of 91 treatments. Examples of objective responses are depicted in Figures 1, 2, and 3. Figure 1 shows a chest x-ray film of patient 10, a 35-year-old man who underwent an exploratory thoracotomy for operable but not resectable, large-cell undifferentiated lung cancer. The patient received two vaccine treatments, with the subsequent disappearance of the lung nodule and improvement of mediastinal widening, both on chest x-ray film and computed tomography (CT). The patient was then treated by his referring physician with chemotherapy and irradiation to consolidate the remission, but the disease recurred five months later.

Figure 2 shows the computed tomograms of patient 29, a 62-year-old man who had an excisional biopsy of pulmonary nodules diagnostic of metastatic melanoma. In addition to pulmonary metastases, the patient had noticed a 1-cm nodule in the right thigh shortly before the initiation of vaccine treatment. The pulmonary disease showed regression, the thigh mass disappeared, and the patient had a reduction of serum concentrations to the monoclonal antibody CA 125 from 91 to 33 ng per ml eight weeks following the start of treatment. The patient then showed progressive disease in the lung and also brain metastases after six months.

Figure 3 shows CT scans taken of a 45-year-old woman (patient 32) who presented with an atrial myxoma invading the great vessels. After surgical resection she was treated with infusional chemotherapy with doxorubicin (Adriamycin, Adria Laboratories) hydrochloride, but hepatic metastases developed. She received three cycles of autologous irradiated tumor cell vaccine as per the methods described for series 3 patients. The follow-up scans five weeks later showed disappearance of several vaguely demarcated liver lesions and reduction of a major lesion from 4 by 4 cm to 1.5 by 1.5 cm. New lesions were identified on the skin and also in the lungs, but the hepatic lesions regressed.

Patient 1 had multiple vaguely defined pulmonary infiltrates, which showed more than 50% regression simultaneous with the development of brain metastases. Patient 14 had an exploratory laparotomy with partial resection of a retroperitoneal mass, not identifiable on computed tomography. Signs of a bowel obstruction developed 14 weeks later, again without tumor visible by CT scan. At an operation the patient had tumor obstructing the distal small bowel but the surgeon saw that the original tumor mass was more than 50% reduced.

Patient 17 had a resection of one of several large breast masses palpable and visible on mammography. After three vaccine treatments, the mass adjacent to the resected tumor appeared to enlarge and was resected. Interestingly, only necrotic debris was obtained. When the patient relapsed 68 weeks later, there was a large, palpable, and darkly pigmented nodule in the region from which a biopsy was previously taken, as well as several other areas in both breasts.

The median survival for all patients was 36 weeks. We note that occasionally a patient had an unusually long survival. Patient 12, with recurrent renal carcinoma in the chest wall, showed a greater than 25% increase in the size of the tumor; he was subsequently treated with a regimen of interferon, without significant benefit, then radiation therapy. Lung metastases also developed. Nonetheless, he remains ambulatory and reasonably active 299 weeks after the autologous intralymphatic vaccine therapy started. Patient 15 had progressive liver metastases on a CT scan after three treatments with autologous irradiated tumor cells and was treated with two courses of intrahepatic chemotherapy with mito-

Figure 1.—Left, A chest x-ray film of patient 10 before vaccine therapy shows a lesion (arrow) in the left hilum. Right, After 2 cycles, the left hilar lesion has disappeared.

mycin and fluorouracil. He has declined further medical evaluation but continues to work more than 169 weeks since treatment was initiated.

Direct toxicity was limited to an occasional local infection, rarely fever or other systemic reactions. There were no instances of regional adenopathy, nor did any patient have symptoms of immediate hypersensitivity. There was no evidence of tumor enhancement or acceleration of the clinical course. Wound infections and difficult cannulations were seen primarily in series 1 patients (three patients each). Fever was identified briefly in one patient, who received antipyretics and had a normal temperature after 24 hours. One patient (number 8) with colon carcinoma and extensive pelvic tumor had a self-limited deep vein thrombophlebitis in the contralateral leg; this condition required admission to hospital and anticoagulation but resolved without sequelae. The patient had no further complications until her demise from progressive distant metastases 32 weeks later. Another patient (number 13) with renal carcinoma died suddenly at home; at autopsy a pulmonary embolus was identified. This event is not unexpected in this population, but there is a possibility that the cutdown procedure, requiring immobilization on a guerney for 60 to 120 minutes, contributed to its development. Nonetheless, patients in the later series were not similarly affected.

Figure 2.—Top, A computed tomogram at the start of treatment in patient 29 shows a large pulmonary nodule (arrow) in the left posterior lung. Bottom, A computed tomogram taken 60 days later shows regression of the nodule in the left posterior lung.

Discussion

This report confirms the clinical interest of active specific immunotherapy using autologous cells through intralymphatic injection. The original report by Juillard and colleagues in 1978 showed that tumor vaccines could elicit regressions of metastatic disease,[2] a controversial notion at a time when it was widely held that immunologic mechanisms would be ineffective except for subclinical, micrometastatic disease.[1] We describe here objective regressions in 7 of 32 patients studied. It should be noted that these very ill patients all had tumors for which effective or even palliative therapy is marginal. The responses have been limited and incomplete, however, and the problem of mixed responses is disappointing. McCune and co-workers, in a study of renal carcinoma, interpret mixed responses as a strong argument for the polyclonality of tumors.[15] The response of patient 32 is encouraging, even if there was growth of tumor at other sites, given the severity of liver involvement and the rarity of regressions under these circumstances.

In evaluating the population for possible prognostic factors, we note that all of the responding patients were younger than 65 and most had performance status scores of 2 or less. The mean age of the seven responders, however, was 50, and that age was not appreciably different from that of the group as a whole (mean age, 51.3 years).

Previous chemotherapy was not a contraindication to a response, contrary to the experience of Weisenburger and colleagues.[16] That study, albeit involving allogeneic tissue

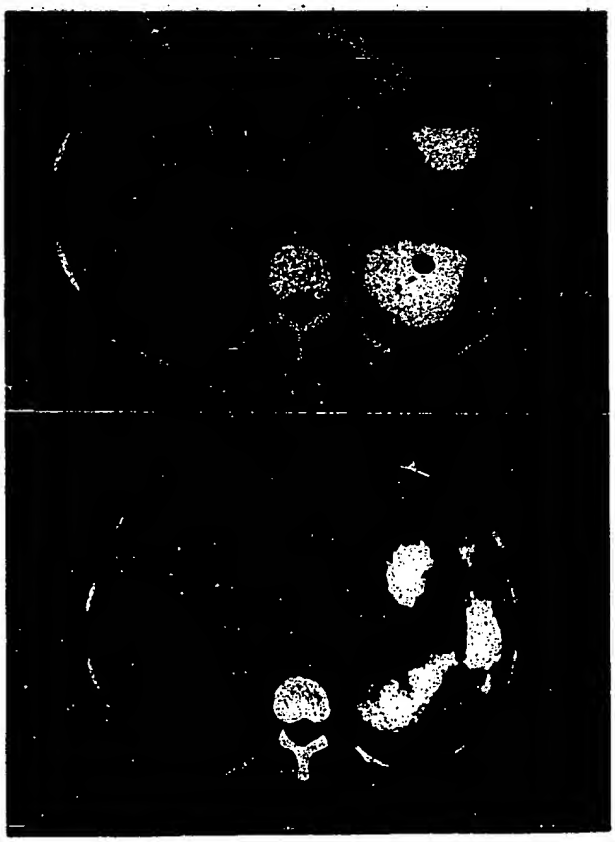

Figure 3.—Top, A computed tomogram taken of patient 32 shows an atrial myxoma (arrow) invading major vessels. Bottom, 70 days later, there is regression of the tumor nodule in the anterior lobe of the liver, with regeneration of liver tissue locally and in the posterior area of the right lobe.

culture cells, identified responses in 9 of 34 patients when intralymphatic therapy was the first method used but no responses in 13 patients who had received previous chemotherapy.[16] In contrast, five of our seven responders had previous chemotherapy.

Although we describe an evolving strategy for implementing active specific intralymphatic immunotherapy, the role of cyclophosphamide and of pretreatment of the tumor cells with cholesteryl hemisuccinate is still unclear. Skornick and associates reported responses in 7 of 21 patients treated with cholesteryl-modified autologous tumor cells.[17] This process did not amplify the response rate in our study. The incidence of clinical responses is not meaningfully different among the three series: 3 of 13 (23%) in series 1, 2 of 7 (29%) in series 2, and 2 of 12 (17%) in series 3.

The method and experience described suggest the existence of host responses that can have potentially useful effects against disseminated malignancy. Attempts to identify the existence of relevant serum antibodies, described by Ahn and co-workers[18] and by Fareed and colleagues,[19] have been unrewarding to date, both in our own laboratory and in an investigation by Karen G. Barnett, PhD, Hybridtech, Inc, San Diego (oral communication, September 1987). We have previously reported an initial impression that intralymphatic immunotherapy produces substantial augmentation of the CD4+ T-cell phenotype.[9,11] A further analysis indicates that this impression is confirmable,[20] and, notably, the more substantial increases of this subset also correlated significantly with the clinical response. Immunologic studies in progress may uncover or clarify further the underlying host-defense mechanisms and will be further evaluated in a separate report.

Our experience suggests that the method of intralymphatic immunotherapy is reasonably safe and technically feasible. It is hard to explain the regressions observed by mechanisms other than host immune responses engendered by the stimulus of the intralymphatic vaccine; understanding this process and modifying it to provide better and more durable responses remain a compelling challenge for further work.

Although one of us (C.L.W.) had previously initiated a similar program at a major academic center, we had some initial concerns about the feasibility of implementing this novel investigation at a community hospital. These concerns were not manifest in reality, and the community support of the program was gratifying. How best to further evaluate the method and to introduce it into clinical practice deserves attention. We are currently engaged in a clinical trial to evaluate a possible role for interleukin 2 in conjunction with this technique of active specific intralymphatic immunotherapy.

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Exhibit 13

C-217*

AUGMENTATION OF DELAYED-TYPE HYPERSENSITIVITY (DTH) TO TUMOR-ASSOCIATED ANTIGENS BY TREATMENT WITH AUTOLOGOUS TUMOR CELL VACCINE PRECEDED BY CYCLOPHOSPHAMIDE (CY). David Berd, Henry Maguire and Michael Mastrangelo. Fox Chase Cancer Center, Philadelphia, PA 19111, Hahnemann Medical College, Philadelphia, PA 19102

CY pretreatment augments the development of DTH to a primary exogenous antigen in patients with advanced cancer. In an ongoing study, we are testing the hypothesis that CY can augment DTH to tumor associated antigens as well. We obtained tumor tissue from patients with advanced cancer who had an easily resectable metastatic deposit. Tumors were dissociated with collagenase and DNase and the cells were cryopreserved in human serum. Patients were skin-tested by i.d. injection of 10^6 live, autologous tumor cells (TC) and also with 3 x 10^6 similarly-cryopreserved blood mononuclear cells (MNC); DTH responses (mean diameter induration) were measured at 48 h. Two weeks later they were given CY 300 mg/M^2 IV. Three days after CY, patients were injected i.d. with a vaccine, consisting of TC (10-30 x 10^6 live cells) mixed with 0.1 ml Glaxo BCG. Seventeen days later, they were skin-tested again; pre- and post-treatment DTH responses were compared. So far, 6 patients have been entered on the study (5 melanoma, 1 breast Ca). Five have completed pre-treatment skin testing and the DTH responses (mm) were: TC - 5,0,4,0,6; MNC - all 0. Two patients have completed at least one immunization and testing. In patient #1, DTH to TC increased after treatment from 5 to 14 mm. Patient #2 had no DTH to TC before treatment, but had a 12 mm response after treatment. Mechanically dissociated TC elicited a response similar to that elicited by enzyme-treated TC. CY vaccine did not augment DTH to MNC. Thus, autologous TC-BCG vaccine preceded by CY can result in development or augmentation of DTH to tumor-associated antigens.

C-218*

COMBINED MODALITY THERAPY OF ADVANCED NODULAR LYMPHOMAS (NL) THE ROLE OF NONSPECIFIC IMMUNOTHERAPY (MBV) AS AN IMPORTANT DETERMINANT OF RESPONSE AND SURVIVAL. S.Kempin, C.Cirrincone, J.Myers, B. Lee III, D.Straus, B. Koziner, Z.Arlin, T.Gee, R.Mertelsmann, C.Pinsky, E.Comacho, L.Nisce, L.Old, B.Clarkson, H.Oettgen, Memorial Sloan-Kettering Cancer Center, New York, N.Y. 10021.

Since June 1976 56 patients (pts) with NL (Stages II-IV) were treated with the NHL-4 protocol, consisting of two sequential regimens: I-Thiotepa, Vincristine, Chlorambucil and Prednisone (6-12 cycles) followed by II-Cyclophosphamide, Adriamycin, Melphalan and Prednisone (4 cycles) (ASCO 22:514, 1981). Radiotherapy (RT) was administered to initial areas of bulky disease at the conclusion of chemotherapy or to nodal/extranodal sites responding only partially to chemotherapy. MBV, a heat killed preparation of S.pyogenes and Ser.marcescens, was administered in a randomized fashion, subcutaneously, one week prior to each chemotherapy cycle, to 26 pts. MBV was administered up until the completion of regimens I and II, then discontinued. The overall complete (CR) and partial response rates were 62% and 32% respectively. The CR rate of vaccinated patients was 85% vs. 44% for nonvaccinated pts. The survival duration of pts treated with MBV is significantly better than nonMBV pts (P=.029); however, with a median follow up of 34.3 months no plateau has yet been reached for either group. Relapses have occurred in 20% of MBV-CR pts vs. 42% of nonMBV CR pts. Of the 10 CR pts who have relapsed, only 5 pts have received RT and all relapses occurred in nonirradiated previously "uninvolved" sites. Including PR pts, 83% relapsed or progressed in previously involved unirradiated sites. This trial suggests that MBV may have a significant therapeutic role in this combined modality approach. Whether longer term maintenance immunotherapy or more aggressive radiotherapy (i.e. total nodal) should be used can only be determined by long term comparative randomized trials.

C-219

ACTIVE SPECIFIC IMMUNOTHERAPY IN MALIGNANT MELANOMA. ECOG PILOT STUDY PE-680. J.E. Harris, A.C. Hollinshead, B. Fuller, and S. Nisius. Rush Medical College, Chicago and Geo. Washington U. Med. Ctr., Washington.

Sixteen patients (pts) with disseminated malignant melanoma who had failed at least one course of chemotherapy were treated by intradermal vaccinations with 0.2 ml of melanoma cell membrane extracted tumor-associated antigens (TAA) + 0.2 ml Freund's Complete Adjuvant (FCA) given monthly up to 3 times. TAA was purified from a soluble membrane pool obtained from melanoma cells metastatic to lung and liver. TAA was obtained after gel filtration and separation by preparative polyacrylamide gel electropheresis (PAGE) and were analyzed by discontinuous-gradient PAGE. Of 15 evaluable pts. (10 males, 5 females; median age 58 years (yrs), range 32-70 yrs.) 7 progressed following 1 vaccination dying 3.0, 0.5, 1.0, 2.5, 5.0, 4.0 and 0.5 months (mos.) later; 4 pts progressed following 2 vaccinations; 3 dying 4.5, 1.0 and 2.0 mos. later, with 1 currently alive at 5.0 mos.; 4 pts progressed following 3 vaccinations; 3 dying 3.0, 5.5 and 1.5 mos. later with 1 currently alive 5.5 mos. later. One partial remission of disease (complete regression of soft tissue skull metastasis with no change in inguinal node metastatic disease) was seen following the second vaccination in a pt. who then relapsed and died 7 weeks after the 3rd vaccination. There was no relationship between tolerance of, or ability to complete the 3 vaccination regimen and (i) performance status, (ii) extent and nature of metastatic disease, (iii) amount of prior cytotoxic drug therapy. Patients receiving 3 vaccinations had longer disease-free intervals between primary resection and tumor recurrence than did those receiving 1-2 vaccinations. Toxicity: transient pain at sites of vaccination and temperature elevation (99-100°F) on the days of vaccination. All vaccination sites ulcerated; none become infected; all crusted over. This Phase I study establishes that extracted tumor antigen in FCA may be given to malignant melanoma pts. with tolerable morbidity. It has antitumor activity and may be useful for immunoprophylaxis in the adjuvant setting.

C-220

EFFECT OF CHEMOTHERAPY ON MONOCLONAL ANTIBODY-DEFINED LEUKOCYTE SUBSETS IN SOLID TUMOR CANCER PATIENTS. Donald P. Braun, Jules E. Harris and Samuel G. Taylor III. Rush Medical College, Chicago, Illinois.

Percentages and numbers of T cells, monocytes and T cell subsets were measured in peripheral blood mononuclear cells (PBMC) from solid tumor cancer patients receiving chemotherapy. The patient population consisted of: 1 patient with breast cancer who received adjuvant chemotherapy with melphalan and 5-fluorouracil; 8 patients with non-small cell lung cancer who received mitomycin C, cisplatin and vindesine, 3 patients with small cell lung cancer who received cytoxan, adriamycin and VP-16, 1 patient with melanoma who received methyl-CCNU, and 1 patient with colon carcinoma who received cytoxan, oncovin and methotrexate. The following leukocyte subsets were enumerated: (i) T cells by sheep erythrocyte rosetting, (ii) T cells by OKT.3 monoclonal antibodies, (iii) helper/inducer T cells by OKT.4 monoclonal antibodies, (iv) suppressor/cytotoxic T cells by OKT.8 monoclonal antibodies, (v) monocytes by latex ingestion and (vi) OKM.1 + cells by monoclonal antibodies. Levels of phytohemagglutinin-induced (PHA) DNA synthesis were measured to determine whether drug-induced changes in leukocyte subsets were associated with changes in immune function. In all of the patients studied, absolute numbers of lymphocytes and E rosetting cells declined following drug treatment; there was no difference in the extent of depression or rate of recovery in any T cell subset. The percentage of latex-ingesting PBMC and OKM.1 + cells were found to decline in 6 patients following their drug treatment. PHA responsiveness in PBMC from 9 to 10 drug treated cancer patients improved to at least 150% of pretreatment levels of function following therapy. That was associated in 4 instances with a decline in percentages of latex ingesting cells and OKM.1 + cells but was never associated with selective changes in T cell subsets. Combination chemotherapy does not differentially affect the percentage of T cell subsets but may sometimes depress monocyte percentages in the PBMC of treated cancer patients.

Exhibit 14

NZ 406 ENHANCED EXPRESSION OF MHC MOLECULES AND STIMULATION OF AUTOLOGOUS TUMOR INFILTRATING LYMPHOCYTES FOLLOWING TRANSDUCTION OF MELANOMA CELLS WITH IFN-γ GENES, Masahiro Ogasawara and Steven A. Rosenberg, Surgery Branch, NCI, NIH, Bethesda, MD 20892

Gene Therapy for cancer is being tested in clinical trials by using tumor infiltrating lymphocytes (TIL) or tumor cells modified by the insertion of genes coding for IL-2 or TNF-α. In the present study, we investigated the feasibility of transducing human tumor cells with genes coding for IFN-γ or IFN-α, which are two other cytokines that can enhance host antitumor immune responses.

Tumor cells from twelve melanoma and two renal cell carcinoma patients were transduced with IFN-γ retroviral vectors. In both IFN-γ secreting and non-secreting tumor lines, the cell surface expression of HLA class I and class II molecules increased following transduction. However the magnitude of the increase in MHC expression appeared to be greater in tumor lines secreting IFN-γ. Northern blot analysis showed IFN-γ transcripts only in IFN-γ transduced cells. The amount of RNA transcribed correlated roughly with IFN-γ secretion.

Two melanoma cell lines were successfully transduced with an IFN-α retroviral vector. Melanoma cells transduced with the IFN-α gene transcribed IFN-α RNA and secreted large amounts of IFN-α. In contrast to cells transduced with the IFN-γ gene, the expression of HLA class II molecules was not increased in IFN-α -transduced cells.

Finally, we tested the ability of HLA.DR+ melanoma cells, which had been transduced with the IFN-γ gene, to stimulate specific cytokine release by autologous CD 4+ TIL. Both GM-CSF and IFN-γ were secreted when the lymphocytes and tumor cells were cultured together but not when they were cultured alone or with control tumor cells. These results suggest that the HLA.DR molecules newly expressed on the transduced cells promoted antigen presentation and T cell responses against the transduced tumor cells. The insertion of IFN-γ genes into melanoma cells may be useful either for active immunization against melanoma or for the generation of TIL to be used in adoptive immunotherapy.

NZ 407 RETROVIRAL MEDIATED GENE TRANSFER OF IL-2 DECREASES TUMORIGENICITY IN MURINE B CELL LYMPHOMA. Orchard P.J.[1,2], Katsanis E.[1], Gorden K.[1], May C.[1], McIvor R.S.[2] and Blazar B.R.[1,2] [1]*Department of Pediatrics, Division of Bone Marrow Transplantation and* [2]*Institute of Human Genetics, Department of Laboratory Medicine and Pathology, University of Minnesota, 55455*

The potential to increase immune responsiveness against otherwise minimally immunogenic malignancies by the transfer of cytokine genes into tumor cells has been of great interest. We have examined the effect of endogenous production of Interleukin-2 (IL-2) on BDL-2, a murine B cell lineage lymphoma, by transducing BDL-2 with a retrovirus (LIL2SN) we have constructed containing the human IL-2 cDNA under transcriptional regulation of the Moloney long terminal repeat, the SV40 internal promoter and the neomycin phosphotransferase gene. BDL-2 clones transduced with LIL2SN were isolated by limiting dilution in G-418, and have been shown to secrete 0.2 - 88.5 U IL-2/10^6 cells/mL/24 hours (mean 29.5 U/mL) by ELISA. No changes in phenotype (MHC class I, CD2, CD5, B220, ICAM-1 or surface IgG) or variation in the rate of proliferation were observed following retroviral transduction and expression of IL-2. Intravenous (iv) or intraperitoneal (ip) injections of this IL-2 secreting BDL-2 clone in syngeneic Balb/c mice resulted in significantly increased median survival time (MST) when compared to controls ($p< 0.03$ and $p< 0.001$, respectively). Immunization with irradiated IL-2 secreting tumor cells subcutaneously (sc) resulted in enhanced survival ($p< 0.0002$) following live tumor challenge with the parental BDL-2 line 14 days later. In a minimal residual disease model, iv injection of 10^5 BDL-2 cells followed on day 12 by sc administration of 10^7 irradiated IL-2 secreting cells resulted in a significant ($p< 0.01$) improvement in MST. In vitro ^{51}Cr release assays demonstrated sensitivity of BDL-2 to both natural killer (NK) populations and activated cytotoxic T cells. In vivo depletions of $CD4^+$ cells (RL 172 antibody) $CD8^+$ cells (2.43) and NK cells (anti-asialo GM_1) were performed. Depletion of $CD8^+$ and NK cells resulted in decreased survival in mice inoculated with IL-2 secreting tumor cells iv, compared to controls and to mice depleted of $CD4^+$ cells. This implicates both cytotoxic T cells and NK cells as important in the resistance of Balb/c mice to a IL-2 secreting BDL-2 tumor line in vivo.

NZ 408 EXPRESSION OF SYNGENEIC MHC CLASS II GENES IN MELANOMA CELLS INHIBITS METASTATIC DISEASE. S. Ostrand-Rosenberg and Noelle Patterson, Dept. of Biology, University of Maryland, Baltimore, MD 21228.

Previous studies have established that transfection of syngeneic MHC class II genes into constitutively class II^- mouse sarcoma cells produces an immunogenic tumor (SaI/A^k) which is rejected by the autologous host, and which effectively immunizes the host against a challenge of wild type class II^- tumor. We have hypothesized that the SaI/A^k transfectants induce protective immunity because they function as antigen presenting cells (APC) for endogenously synthesized tumor peptides, and thereby stimulate tumor-specific T_h cells, by-passing the need for professional APC. In the present study we demonstrate that immunization with MHC class II gene transfected tumor also protects the autologous host against subsequent metastatic disease. The C3H-derived (*H-2*^{k}) K1735 melanoma gives high levels of spontaneous (subcutaneous inoculation) and experimental (intravenous inoculation) metastastases in syngeneic C3H mice. In order to test the protective potential of MHC class II^+ K1735 cells, wild type K1735 tumor cells were transfected with syngeneic A_a^k, A_b^k MHC class II genes, and/or neo^R gene, and 3 clones expressing high levels of I-A^k molecules selected (K1735/A^k clones). At tumor doses ranging from 10^3 to 5 X 10^5 i.v., the class II^+ transfectants give 5-10 fold fewer lung metastases than their wildtype class II^- or neo^R alone counterparts. C3H mice inoculated subcutaneously with class II^+ K1735 cells have significantly lower frequencies of primary tumors and spontaneous metastases, as compared to mice receiving wild type K1735 inocula. We have also tested the ability of K1735/A^k cells to immunize against wild type tumor. Autologous C3H mice immunized with fixed class II^+ K1735 cells and challenged i.p. 1-3 months later with wild type K1735 tumor have greatly reduced primary tumor growth and spontaneous metastasis formation relative to naive, unimmunized recipients. These studies indicate that transfection and expression of syngeneic MHC class II genes significantly reduces the metastatic potential of a mouse melanoma, and provides an immunization stategy for protecting against subsequent metastatic disease.

NZ 409 THE RETROVIRAL VECTOR MFG ALLOWS HIGH EFFICIENCY TRANSDUCTION OF HUMAN PROSTATE CANCER CELLS: IMPLICATIONS FOR GENE THERAPY OF PROSTATE CANCER. Martin G. Sanda, Sujatha Ayyagari, Liz Jaffee, Drew M. Pardoll, Richard C. Mulligan, and Jonathan W. Simons, Oncology Center and Brady Urological Institute, Johns Hopkins Hospital, Baltimore, MD, and the Whitehead Institute, MIT, Cambridge, MA.

We addressed the feasibility of gene therapy for human prostate cancer using MFG, an amphotrophic and replication defective retroviral vector lacking the gag, pol, and env genes. First, conditions optimizing transduction efficiency using MFG-lacZ (containing the gene encoding beta-galactosidase) were identified with transduction of four long term human prostatic adenocarcinoma cell lines. DEAE-dextran dose and virus-target coincubation titration demonstrated optimum transduction efficiency at 80ug/ml of DEAE-Dextran with 5 hours of target exposure to viral supernatant. Using these conditions, 21-82% transduction efficiency was achieved. We then used these conditions to transduce prostate cancer cells from patients undergoing radical prostatectomy. Cultured cells derived from surgical specimens were confirmed to be of prostatic epithelial origin by immuno-histochemical detection of luminar epithelium specific cytokeratin 18, and by detection of Prostate Specific Antigen (PSA) secretion by these cells. 200 - 300 fold expansion was achieved in vitro prior to senescence of cultured cells. Efficient transduction by MFG-lacZ of such primary culture prostate cancer cells from 7 consecutive patients (transduction efficiency range, 4.3% to 50%; median, 15.2%) demonstrates the feasibility of using MFG in genetic therapy for prostate cancer.

Exhibit 15

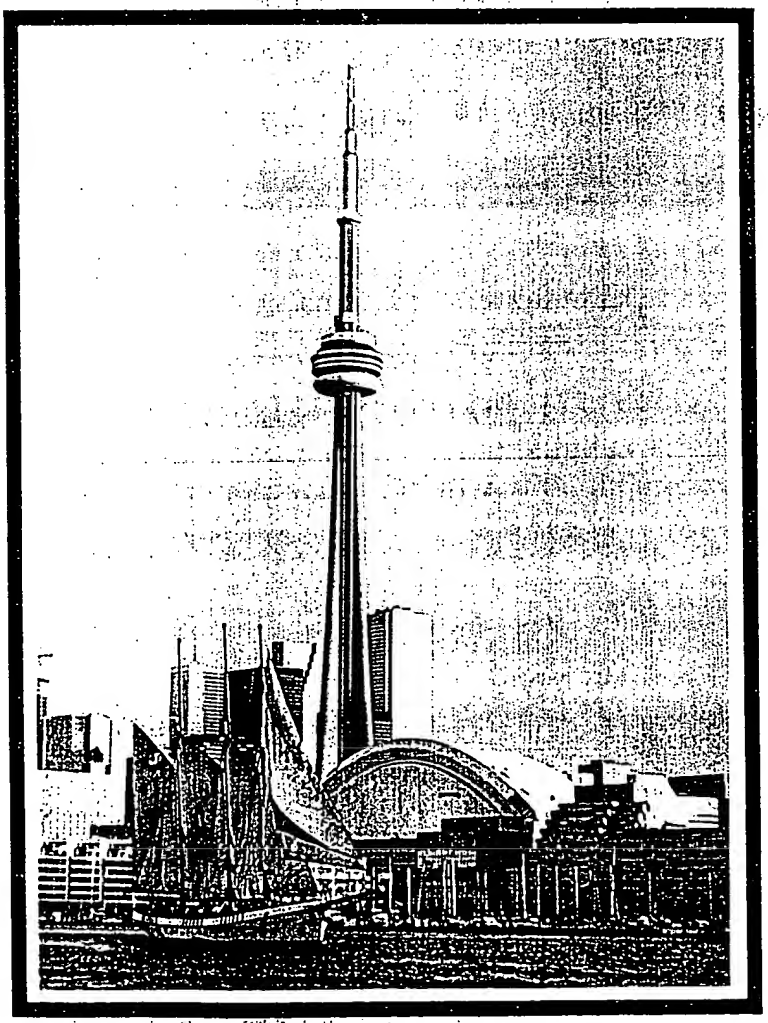
Volume 145 April 1991 Number

The Journal of UROLOGY®

Official Journal of the American Urological Association, Inc.
Founded In 1917 By Hugh Hampton Young

AUA EIGHTY-SIXTH ANNUAL MEETING
JUNE 2-6, 1991

METRO TORONTO CONVENTION CENTRE — TORONTO



THE CN TOWER

(Photo courtesy of Metropolitan Toronto Convention & Visitors Association)

Supplement to The Journal of Urology

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THE ENDOCUT NEEDLE: ENDOSCOPIC CORE BIOPSY WITHOUT NEEDLE TRACK SEEDING. *Chris J Parker, *Brian R Birch and Ronald A Miller. London, UK. (Presentation to be made by Dr Birch).

The Endocut Needle (Cook Urological) is the first spring loaded, automatic, core biopsy needle which can be passed via the instrument channel of a cystoscope, nephroscope or laparoscope. Biopsy procedures can be carried out under sedation and local anaesthesia as day-cases. A prospective study of 10 consecutive patients requiring prostatic biopsy was carried to assess the accuracy and acceptability of this new instrument.

After preliminary cystoscopy prostatic biopsies were taken x2 with the endocut needle (EN) per urethram (direct vision) and x2 with a Trucut needle (TN) per rectum (blind). Four patients subsequently underwent prostate resection (TURP) and the material so obtained was compared with that from earlier EN and TN biopsy.

Prostatic tissue was obtained in all (20/20) cases of EN biopsy but fat/fibrous tissue only was reported in 3/20 TN biopsies. EN biopsy histology was compatible with TURP material in all 4 cases. TN biopsy agreed in just 3/4 cases missing one diagnosis of carcinoma. EN biopsy was preferred to TN biopsy by all patients

It is evident that EN biopsy is accurate (an echogenic needle is available where ultrasound localisation is required), preferred by patients, reduces the risk of sepsis and operator injury and provides core samples suitable for histological examination. In the prostate this is achieved without the risk of needle-track seeding. Synchronous endoscopic and histological assessment of prostate (or bladder) tumours can then be performed as an out-patient.

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A NOVEL MULTIDRUG RESISTANCE-1 RELATED PRODUCT SYNTHESIZED EXCLUSIVELY DURING THE REGRESSION OF THE RAT VENTRAL PROSTATE GLAND. Ilan J. Leibovitch, Carl A. Olsson, Benad Goldwasser and Ralph Buttyan, New York, NY and Tel Hashomer, Israel (Presentation to be made by Dr. Leibovitch).

The rat ventral prostate gland is an outstanding model to characterize the molecular pathway by which programmed death (apoptosis) is activated. Castration rapidly induces apoptotis in prostatic epithelial cells. As with other cell activities, apoptosis requires the synthesis of proteins and RNA; thus we can surmise that some product(s) made after castration is needed for the cell to die. We have already described constitutive gene products that are induced during regression. Other gene products have been identified (sulfated glyco-protein-2 and the Yb1 subunit of glutathione S-transferase) that are so intensely induced they seem specific for dying cells. Our studies on the role of multidrug resistance (MDR) genes in the prostate has allowed us to identify another member of this latter category, a gene product related to MDR-1 P-glycoprotein. Low stringency hybridization of Northern blots containing mRNA from regressing ventral prostates shows the intense induction (150-fold) of a 2 kb transcript (compared to 4.9 kb for MDR-1 mRNA). While undetectable in normal ventral prostate, the expression of this mRNA reaches a peak at 3-4 days after castration and declines thereafter, being barely detectable at 1 week. By screening a cDNA library made from 3-day castrate prostate mRNA, we have isolated clones for this gene and sequencing studies should determine its relationship to the normal MDR-1 product. This finding provides another member of the regression-associated products with which to identify genetic elements determining death-specific expression.

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LYMPHOKINE-TRANSFECTED PROSTATE CANCER CELLS GENERATE AN ANTI-TUMOR EFFECT IN VIVO. *D. Branch Moody, *Charles M. Ewing and *William B. Isaacs, Baltimore, Maryland. (Presentation by D. Branch Moody)

We demonstrate an anti-tumor effect of lymphokine-transfected tumor cells against an anaplastic, rapidly growing rat prostate carcinoma. Lymphokine-secreting tumors were prepared by transfecting cDNA for Interleukin 2 (IL2) and Interleukin 4 (IL4) into the MAT LyLu (MLyLu) subline of the Dunning rat prostate carcinoma. Injection of the $7x10^5$ MLyLu cells produced lethal tumors in 100% of control rats; whereas, the same number of IL2-transfected MLyLu cells produced no tumors in 32 animals. The lymphokine-transfected cell therapy gave partial protection against subsequent injections of parental tumor. Ten of 20 animals injected with parental tumor 2-3 weeks after treatment showed no tumor growth. Animals suffered no readily apparent toxicity from the treatment. Currently, experiments are underway to further define the duration of the anti-tumor effect, maximal tumor burden which can be treated with IL2-transfected tumor, as well as potential synergy between cells transfected with IL2 and cells transfected with IL4. The results indicate that interleukin-secreting prostate cancer cells result in the rejection of an otherwise lethal prostate cancer cell inoculum and give short lived partial protection against subsequent challenge with parental tumor cells. This work is the first use of this novel lymphokine therapy of prostate cancer in animals.
(DBM supported by American Cancer Society Institutional Research Grant.)

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INVESTIGATION OF RETINOBLASTOMA TRANSCRIPTS IN PRIMARY PROSTATIC ADENOCARCINOMA°. *J.A. Petros, *S.B. Dowton, W.J. Catalona. St. Louis, MO (Presentation by Dr. Petros)

Tissue has been preserved from 60 patients whose prostate was removed for clinically localized prostate cancer. Intraoperative selection of tumor was made, and specimens were frozen immediately in liquid nitrogen. Messenger RNA was purified by cesium chloride ultracentrafugation and by oligo dT resin affinity chromatography. Of 12 specimens investigated to date, 4 have shown absence of retinoblastoma (RB) mRNA when a cDNA probe which hybridizes with the 5' region of the RB gene (kindly provided by Dr. Takahashi, Baylor College of Medicine, Houston, TX) was used in RNA blot analysis. Integrity of mRNA and uniform RNA application to the gel were documented by re-hydridization with an actin probe. These results have not yet been reconfirmed by reanalysis of the same samples. If verified, these data suggest that the mRNA encoding the RB protein is partially or wholly absent in some primary human prostatic adenocarcinomas. Investigation is currently in progress to determine if the 3' region of the RB gene is transcribed in these tumors, or if the mRNA is completely absent. Future investigations will address the genomic mutation(s) responsible for abnormal transcription, as well as correlation with tumor behaviour and patient outcome.

°Supported by NIH Surgical Oncology Training Grant #1T32CA09621-02 and the Urological Research Foundation of Washington University

Exhibit 16

EXPRESS MAIL CERTIFICATE

Date 5/29/01 Label No. EK706741126US

I hereby certify that, on the date indicated above, this paper or fee was deposited with the U.S. Postal Service & that it was addressed for delivery to the Assistant Commissioner for Patents, Washington, DC 20231 by "Express Mail Post Office to Addressee" service.

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PATENT TRADEMARK OFFICE

1225/0C674

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: David BERD

Serial No.: 08/203,004 Art Unit: 1642

Filed: February 28, 1994 Examiner: Susan UNGAR

For: COMPOSITION AND METHOD OF USING TUMOR CELLS

DECLARATION OF DONALD P. BRAUN, PH.D.
UNDER 37 C.F.R. § 1.132

Hon. Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, Donald P. BRAUN, hereby declare and state as follows:

1. I am a citizen of the United States of America and am more than 21 years of age.

2. I presently hold the title of Administrative Director of the Medical College of Ohio Cancer Institute and Professor of Surgery at the Medical College of Ohio, 3120 Glendale Avenue, Toledo, Ohio, where I have been employed since 1999. Prior to this position, I held the positions of Director, Scientific Program Development and Professor of Medicine and Immunology/Microbiology at the Rush Cancer Institute, Rush Medical College, Chicago, Illinois. I hold a Ph.D. and M.S. degrees from the University of Illinois at the Medical Center, Chicago, and a B.S. from the University of Illinois, Urbana. I have over 25 years research experience in immunology, microbiology, and oncology, particularly cancer immunology. My qualifications are set forth more fully on the copy of my *Curriculum Vitae,* attached as Exhibit A.

3. My only connection with Avax Therapeutics, Inc. ("Avax"), is as a clinical researcher. I understand Avax has licensed certain patents and patent applications by Dr. David Berd (solely or with others) related to haptenization of tumor cells to generate an effective anti-tumor immunotherapy ("technology") from Thomas Jefferson University. I am not an employee or shareholder of Avax.

4. I know Dr. David Berd professionally. However, we have not collaborated on any research.

5. The law firm of Darby & Darby, attorneys for Applicant, has retained my services as an expert in connection with prosecution of these patent applications. In connection with these services, I attended and participated in a personal interview with the Examiner on

January 5, 2001. The law firm is compensating me for my services. Thus, I have no personal interest in Avax or the patent applications.

6. I have read and am familiar with Berd et al., Proc AACR 1989;20:382 (hereinafter "Berd 1989"; a copy is attached as Exhibit B). In particular, it is my understanding that the claims of the above-identified application have been rejected in part because the Examiner believes that Berd 1989 teaches a successful method of inducing an antitumor response comprising regression of a metastatic melanoma tumor by administering cyclophosphamide prior to autologous, irradiated, DNP-conjugated melanoma cells in combination with BCG.

7. In my view as one of skill in the art in this field, the Berd 1989 abstract does not describe successfully treating melanoma tumors with a haptenized melanoma tumor cell immunotherapy vaccine. The abstract, like most of the abstracts presented at the AACR meetings, optimistically reports preliminary observations from a new protocol. Because the abstract omits certain details, and because by its own terms the results are preliminary, one of ordinary skill in the art would not be able to conclude from this Abstract that one could effectively treat melanoma, much less any other type of cancer. Nothing in the Berd 1989 abstract suggests that this approach addresses fundamental questions of tumor vaccination (*e.g.*, as posed in a 1993 review on tumor vaccination written by myself and Jules Harris, M.D. for the Biotechnology Journal (Volume 1, No. 3), entitled "Cancer-Concept to Clinic" (Exhibit C)); which type of immune response are most important in a host response to cancer (Exhibit C, p. 28 and Table 1); whether whole cells or extracts should be used (Id., pp. 28-29); whether to use adjuvants or cytokines (Id., p. 29); and whether an

antitumor response would lead to autoimmunity (Id.). Furthermore, with respect to whole cell vaccines, whether to use autologous or syngeneic cells; fresh surgical specimens or cell lines; irradiation; reproducibility; and other factors (Id. P. 29, Table 2). The haptenization protocol of the Berd 1989 Abstract not only fails to address these variables, but also raises a new issue. Consequently, in 1989, one of skill in the art would not have viewed Berd 1989 as establishing an effective protocol for cancer immunotherapy.

8. By way of background, as described during the interview, early work on developing tumor vaccines in animal models yielded successes far beyond the reality for humans. Animals used in these models are typically immunocompetent, and the tumor cell lines (unlike spontaneous tumors) bear one or more strongly immunogenic antigens. Under these circumstances, the ability to generate an immune response cannot be viewed as particularly surprising. Unlike animal models, human cancer patients are typically immunosuppressed, whether from the tumor or chemotherapy. Spontaneous human tumors are weak immunogens. Thus the trick is to determine how to break tolerance and elicit immunity in a human subject. In 1992, Hanna and colleagues proposed one route, albeit based on animal data; but their results were inconclusive (Exhibit C, p. 30). Berd and colleagues offered another approach, pretreatment with cyclophosphamide to inhibit suppressor T lymphocytes (Id.) In the context of these multiple approaches, it was, in 1989, unknown and unknowable whether haptenization was a viable approach to elicit immunity to unhaptenized melanoma cells, much less that the approach could have therapeutic potential.

9. In my view as one of skill in the art in this field, the Berd 1989 abstract does not provide a definitive protocol. The description of the vaccine is ambiguous, stating that 10-25 million cells are used. It does not state if these are given as a single injection or divided into multiple sites, nor does it specify the route of administration (*e.g.*, intradermal, subcutaneous, or intramuscular). It does not specify if the injections are given in proximity to tumor sites or even directly into the tumor site (a location that one familiar with the literature at the time would assume from a reading of this abstract). It does not state how conjugation to DNP was performed or the extent of tumor cell substitution. It does not specify the ratio of tumor cells to BCG microorganisms. It also does not describe the schedule of vaccination beyond stating that vaccine or DNCB sensitization occurred 3 days following low dose cyclophosphamide i.v. administration. The statement "after 2 vaccine treatments (8 weeks)" is totally ambiguous. It is not clear if this represented a point 4 weeks following vaccine #2, 3 weeks following vaccine #2, 2 weeks following vaccine #2, or 1 week following vaccine #2. A vaccination schedule of every 55 days could apply to what is described as readily as any of the other schedules listed above. Hanna and Peters (Cancer Research 1978;38:204-9, attached hereto as Exhibit D) emphasize the critical importance of dose, schedule, route of administration, and ratio of viable tumor cells to BCG organisms in the outcome of autologous tumor vaccines. The Berd 1989 abstract, however, provides none of these details, nor could they be deduced. Without these details, one of ordinary skill would be unable to practice the technology predictably, and furthermore would have little incentive to view this approach as any more promising than a myriad of others.

10. ***There is no indication in the protocol that patients have developed an immune response to unmodified cells.*** The opening statement of Berd 1989 indicates that a previous method practiced by Berd using non-haptenized tumor cells induced DTH to melanoma cells. But in the Berd 1989 abstract, DTH testing was done only with DNP-modified tumor cells or DNP-modified autologous lymphocytes following patient sensitization with topical application of DNCB. The positive reactions described in the Berd 1989 abstract are not surprising given the experience of Fujiwara (J Immunol 1980;124:863-869; attached hereto as Exhibit E), Sherman (J Immunol 1979;123:501-502; attached hereto as Exhibit F), and others using haptens to sensitize hosts against haptenized target cells. However, the vaccine protocol of the invention involving intradermal injection of hapten-modified autologous tumor cells, results in DTH to autologous non-haptenized tumor cells, an event that could not have been anticipated nor expected as a result of what is described in the Berd 1989 abstract or from what was known in the literature.

11. ***There is no convincing indication that the patients described in the Berd 1989 abstract received any clinical benefit.*** The descriptions of inflammatory reactions, CD4 and CD8 infiltration, and fluid accumulation over tumor lesions is no indication of clinically significant tumor regression (defined by those practiced in the art as a greater than 50% reduction in tumor size without concomitant progression in other sites). In fact, the description of lesion changes in the patients would be expected at the time of its publication, since one would presume based on Fujiwara (Exhibit D), that the patients had been sensitized to DNCB and then injected intratumorally with DNP-modified tumor cells. A skilled immuno-oncologist would have presumed that the tumor cell vaccine, which produced the described physical changes in proximity to tumor sites after only

two vaccine treatments, had been administered by intratumoral injection as taught by others (while not practiced by the Berd protocol developed subsequent to the 1989 Abstract). The same outcome would have been seen if the patients had been sensitized to BCG and then injected intratumorally with BCG. Thus, the description of the lesion changes in the 1989 Abstract would be impossible to interpret as indicative of a clinical response to a systemic vaccine. A reader would assume that a clinically meaningful tumor regression, if present, would have been reported in the abstract, and that the absence of such a report represented uncertainty.

12. For all of these reasons, the Berd 1989 abstract does not disclose a method for the successful vaccination of cancer patients using haptenized autologous tumor cells.

13. A final basis for the above statement can be deduced from Exhibit C. As discussed above, this review cites the work by Hanna and Hoover (references 7-9), and the work by Berd et al. employing non-haptenized melanoma cells (reference 10), among a number of hopeful, even promising, research approaches to cancer immunotherapy. Had the Berd 1989 abstract been indicative of a clinically meaningful vaccine methodology, that approach would have been considered in the review as well.

14. I declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

code and that such willful false statements may jeopardize the validity of the instant application or of any patent issued thereupon.

Respectfully submitted,

Date: 5/24/01

Donald P. Braun, Ph.D.

Enclosure:

Exhibit A:	Curriculum Vitae of Donald P. Braun, Ph.D.
Exhibit B:	Berd et al., Proc AACR1989;20:382
Exhibit C:	Braun and Harris, Biotechnol J 1993;1, No. 3.
Exhibit D:	Hanna et al., Cancer Research 1978;38:204-209
Exhibit E:	Fujiwara et al., J Immunol 1980;124:863-869
Exhibit F:	Sherman, J Immunol 1979;123:501-502

Exhibit 17

Adjuvant Active Specific Immunotherapy for Human Colorectal Cancer: 6.5-Year Median Follow-Up of a Phase III Prospectively Randomized Trial

By Herbert C. Hoover, Jr, Jane S. Brandhorst, Leona C. Peters, Mildred G. Surdyke, Yoshiko Takeshita, Juan Madariaga, Larry R. Muenz, and Michael G. Hanna, Jr

Purpose: Patients with colon or rectal cancer were entered onto a prospectively randomized, controlled clinical trial of active specific immunotherapy (ASI) with an autologous tumor cell–bacillus Calmette-Guérin (BCG) vaccine. We investigated whether ASI could improve disease-free status and survival.

Patients and Methods: Ninety-eight patients with Dukes' stage B_2-C_3 colon or rectal cancer were randomized into groups treated by resection alone or resection plus ASI. Eighty patients met all eligibility criteria. All patients with rectal cancer were to receive 50 Gy of pelvic irradiation. Analysis of distribution of survival and disease-free survival was made on all eligible patients until December 31, 1990.

Results: As a single study, no statistically significant differences were detected in survival or disease-free survival for all 80 eligible patients. However, since it was recognized at the outset that there were treatment differences, in that rectal cancer patients were to receive postimmunotherapy radiation, it was considered that a cohort analysis of the colon and rectal cancer patients might be informative. With a median follow-up of 93 months, there is a significant improvement in survival (two-sided $P = .02$; hazards ratio, 3.97) and disease-free survival (two-sided $P = .039$; hazards ratio, 2.67) in all eligible colon cancer patients who received ASI. With a median follow-up of 58 months, no benefits were seen in patients with rectal cancer who received ASI.

Conclusion: This study suggests that ASI may be beneficial to patients with colon cancer.

J Clin Oncol 11:390-399. © 1993 by American Society of Clinical Oncology.

COLORECTAL CANCER continues to be one of the most prevalent malignancies in the United States, with more than 157,000 new cases diagnosed and more than 60,000 deaths occurring each year.[1] Although surgical resection is usually the only curative therapy, established gross metastases and micrometastases commonly frustrate these efforts. Despite improved surgical approaches, patients with transmural extension of tumor and metastases to regional lymph nodes still have 5-year survival rates of only 30% to 50% when treated by operative resection alone. Adjuvant radiation therapy can lower the incidence of local recurrence, but does not control systemic metastasis, the cause of death in many of these patients.[2-4]

Some recently published trials indicate benefit from chemotherapy, especially a combination of fluorouracil (5-FU) and levamisole in Dukes' stage C colon carcinoma,[5,6] and a combination of radiation therapy and 5-FU/methyl-lomustine (CCNU) chemotherapy for rectal carcinoma.[7] However, systemic toxicity and drug resistance continue to limit the usefulness of chemotherapy. New approaches are needed to engender further advances.

Biologic therapy or biologic response modifier therapy has moved beyond the era of nonspecific immunotherapy that laid some of the foundation for today's approaches. Our efforts have focused on an approach that makes use of a patient's own tumor to elicit an immune response. This approach is known as active specific immunotherapy (ASI). There is now extensive evidence that many human tumors have tumor-associated antigens, although assessment of specificity of these antigens remains difficult.[8] In our use of ASI, we are presuming the presence of distinct tumor antigens on tumor cells that are absent on normal cells. We are attempting to activate the host defenses against these antigenic factors, which are theoretically distinctive to each tumor, by increasing the immunogenicity of autologous tumor cells with an immunomodulating adjuvant, bacillus Calmette-Guérin (BCG).

The impetus for this work was the development and biologic characterization of an experimental model that determined the requirements for effective immunotherapy of established tumor. A series of studies in a guinea pig line-10 hepatocarcinoma model[9-15] demonstrated that BCG admixed with syngeneic tumor cells can induce systemic immunity capable of eliminating a limited disseminated tumor burden when the vaccine is carefully controlled for variables such as the number of tumor cells

From the Division of Surgical Oncology, Massachusetts General Hospital, Boston, MA; and Organon Teknika/Biotechnology Research Institute, Rockville, MD.

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0732-183X/93/1103-0003$3.00/0

ratio of viable BCG organisms to tumor cells, viability of the tumor cells, and vaccination regimen.

A five-patient pilot study in 1980 demonstrated that we could successfully dissociate human colorectal cancers into viable cell preparations and that the toxicity of the tumor cell-BCG vaccine was minimal. The principles and procedures of ASI, as learned in the guinea pig hepatocarcinoma model, were then applied in a randomized, controlled trial of patients with colorectal cancer. The decision was made to start this trial in a prospectively randomized, controlled manner to maximize the information achieved from an extraordinarily labor-intensive and expensive trial. The study began at the Johns Hopkins Hospital, Baltimore, MD, in 1981, was transferred to University Hospital, Stony Brook, NY, in 1983, and then to the Massachusetts General Hospital, Boston, MA, in 1986. Accrual ended in November 1988 and follow-up extended to December 1990. The objectives of this study were to determine whether ASI could (1) enhance the delayed cutaneous hypersensitivity (DCH) response to autologous tumor cells, and (2) prolong the disease-free survival of these patients. We have reported[16] that immunized patients showed a significant increase in the DCH response to autologous tumor cells compared with autologous normal mucosa, used as a normal tissue control. Furthermore, no significant DCH responses against autologous tumor or mucosa cells were detected in a control group of nonimmunized patients. The qualitative and quantitative differences in DCH responses to tumor cells and to normal mucosa cells suggest that the immunizations are targeted mainly to tumor-associated antigens, and that tissue-associated antigens play a secondary role.

Correction to the Original Report[17]

In 1985,[17] we reported the preliminary clinical results with a mean follow-up of 28 months. In preparing the follow-up report on that work, we were made aware of oversights in the 1985 report that we now wish to correct. In 1985, we failed to state that there were patients randomized onto the trial who were later excluded as being ineligible. These patients are now listed to allow the reader to account for every patient randomized. In this final audit, eligibility criteria have been more rigidly defined, requiring source documentation for all reported data. Thus, some patients included in the original report have been reclassified as to their eligibility. These changes have resulted in a total of 42 eligible patients at the time of the 1985 interim analysis, rather than the 40 patients reported. The net change of two patients results from the reclassification of six patients. The reclassified patients included two control patients who were later found to be ineligible and four treated patients who were reinstated.

The trial was established as simultaneous studies for colon and rectal cancer patients. In the 1985 analysis, all patients were combined for analysis because of the small number of patients. With the reclassification and a more appropriate data analysis, the major conclusion of the 1985 report is altered in that the apparent advantage of immunotherapy is limited to patients with colon cancer. There is no perceived benefit in rectal cancer patients. On July 31, 1984, of 27 colon cancer patients, 38% of control and 14% of treated patients had recurred and 23% and 0%, respectively, had died. Of 15 rectal cancer patients, 60% of control and 50% of treated patients had recurred and 0% and 10%, respectively, had died. Statistical analysis of such small numbers has little power to detect differences, but the encouraging advantage in the colon cancer patients who received immunotherapy is suggested.

This report updates that clinical trial with a total of 80 eligible patients. It accounts for every patient randomized and elaborates on the methodology used for the entire trial.

PATIENTS AND METHODS

Patient Selection and Randomization

Eligible patients were those with colon or rectal cancers extending through the bowel wall (Gunderson and Sosin[18] stages B_2 or B_3) or with positive lymph nodes (stages C_1, C_2, or C_3) (Table 1) for whom adequate cells from the primary tumor were available. Rectal cancer was defined as any tumor involving or below the pelvic peritoneal reflection. Suitable medical condition to comply with the outpatient treatment protocol was required (Eastern Cooperative Oncology Group [ECOG] performance status 0 or 1). From 1981 until 1983, patients with a history of cancers controlled by surgery, chemotherapy, or radiation therapy greater than 5 years from entry into the study were considered eligible. Since 1983, to avoid any chance of confusing a recurrence from a prior cancer, patients with any prior cancer (except basal or squamous cell skin cancer) have been excluded. The issue of prior malignancies is the only change in the eligibility criteria as the study progressed. Resection of the tumors was performed at Johns Hopkins Hospital, Loch Raven Veterans Hospital, Baltimore City Hospital, St Agnes Hospital, and Greater Baltimore Medical Center, all in Baltimore, MD; University Hospital, Stony Brook, NY; Peninsula General Hospital, Far Rockaway, NY; Northwestern Memorial Hospital, Chicago, IL; and Massachusetts General Hospital, Boston, MA. These studies were approved by each hospital's human studies institutional review board before any patient was entered. Informed consent was obtained from each subject. Although patients were accrued from nine hospitals, 82% of the vaccine preparations were made, and ASI patients treated, under the direct supervision of the principal investigator (H.C.H.) at Johns Hopkins, Stony Brook, and Massachusetts General. The remaining 18% were performed at Northwestern and Peninsula General, by personnel trained and quality-controlled by the principal investigator's staff, thus minimizing

site-to-site variability in vaccine preparation and administration. Figure 1 shows the schema for stratification and randomization. Randomization was centrally controlled at the institution of the principal investigator from a single set of sealed envelopes. Colon cancer patients were randomized separately from rectal cancer patients with substratification within each of the pathologic stages B_2 through C_3. Randomization cards were sequentially drawn in a blinded fashion by a protocol nurse from each category at the time (postoperatively) the patient was determined to be eligible and signed an informed consent form. An initial target sample size was 100 patients.

Surgical and Pathology Quality Control

Operative and pathology reports were reviewed carefully for adequacy of the surgical procedure and accuracy of staging, prompting a direct communication with the surgeon or pathologist in any case where questions developed. Each eligible patient had wide removal of the involved bowel segment, as well as the lymphatic drainage area. Involved adjacent organs were resected en bloc. All patients had histologically proven clear margins. Five patients were equivocal between B_1 and B_2 stages and have been called B_2 for this protocol.

Preparation of Autologous Vaccines From Solid Primary Tumors

After resection, the bowel specimen was taken immediately to the hospital pathology department and opened under sterile conditions. All tumor tissue not required for staging and 2 to 3 g of distal normal colon mucosa were excised, placed in sterile tubes with Hanks' balanced salt solution containing 50 μg gentamicin/mL, and carried immediately on ice to the laboratory for dissociation and freezing using the method reported by Peters et al.[14] The importance of minimizing damage to the metabolic activity of the cell by careful and timely dissociation and optimal cryopreservation has been addressed previously.[14-17]

A sample of the enzyme-dissociated cell preparation was sent to bacteriology for routine culture as previously described[16] or was cryopreserved along with the vaccine and later thawed for bacteriologic testing.

Clinical Protocol

Patients with tumors of the appropriate pathologic stages were randomized postoperatively either to receive the autologous tumor cell-BCG vaccine or to receive no vaccine (Fig 1). Colon cancer and rectal cancer patients were in separate but parallel studies that were identical except that postoperative pelvic irradiation was recommended for all patients with rectal cancer. Before randomization, we attempted to screen all consenting patients by chest x-rays, liver ultrasound or computerized axial tomography scans, and carcinoembryonic antigen (CEA) tests to identify patients with unsuspected metastatic disease. The vaccines were started at 4 to 5 weeks after tumor resection to allow the patient sufficient time to recover from immunologic suppression, which may be induced by anesthesia and surgery. At 3 to 4 weeks after resection, both control and immunized patients were skin-tested with standard recall antigens to evaluate immunocompetence and sensitization to tuberculin purified protein derivative (PPD). When sufficient cells were available, patients were also skin-tested with graded doses (10^4, 10^5, and 10^6) of autologous tumor and mucosa cells. After 1984, only the 10^6 dose was used, as the lower doses proved uninformative. Repeat skin testing with tumor cells and mucosa was performed at 6 weeks, 6 months, and 1 year following vaccination when sufficient autologous material was available as described previously.[16] Repeat recall antigen testing was performed at 6 weeks when possible.

Treated patients received one intradermal vaccination per week for 2 weeks consisting of 10^7 viable, irradiated, autologous tumor cells and 10^7 viable BCG organisms, as determined by colony-forming units. (Fresh-frozen TICE BCG was supplied by [illegible]on Teknik-

Table 1. Characteristics of the ASI Treatment Groups (eligible patients only)

Characteristic	Immunotherapy (n = 41)		Control (n = 39)	
	n	%	n	%
Age, years				
Mean	63		61	
Range	31-87		36-80	
Sex				
Male	27	66	24	62
Female	14	34	15	38
Race				
White	35	85	31	79
Black	5	12	5	13
Other	1	2	3	8
Location of primary*				
Right colon	10	24	11	28
Left colon	14	34	12	31
Rectal	17	41	16	41
Astler-Coller stage				
Colon				
B_2 (transmural)	14	34	11	28
B_3 (adjacent organ)	1	2	2	5
C_1 (+node, not transmural)	2	5	0	0
C_2 (B2 with +node)	6	15	8	20
C_3 (B3 with +node)	1	2	2	5
Rectal				
B_2 (transmural)	4	10	6	15
B_3 (adjacent organ)	0	0	0	0
C_1 (+node, not transmural)	4	10	2	5
C_2 (B2 with +node)	9	22	8	20
C_3 (B3 with +node)	0	0	0	0
Size of primary, cm				
Mean	5.2		5.3	
Range	3.0-10.0		3.0-11.5	
Proximal margin, cm				
Mean	15.4		16.3	
Range	2.4-40		[illegible]	
Distal margin, cm				
Mean	8.6		6.8	
Range	1.4-25		0.5-18.5	
No. of +nodes (stage C only)				
Mean	3.2		3.7	
Range	1-13		1-23	

Abbreviation: +node, positive node.

*Right colon includes bowel from cecum to midtransverse colon, left colon from midtransverse to peritoneal reflection, rectum below peritoneal reflection to anus.

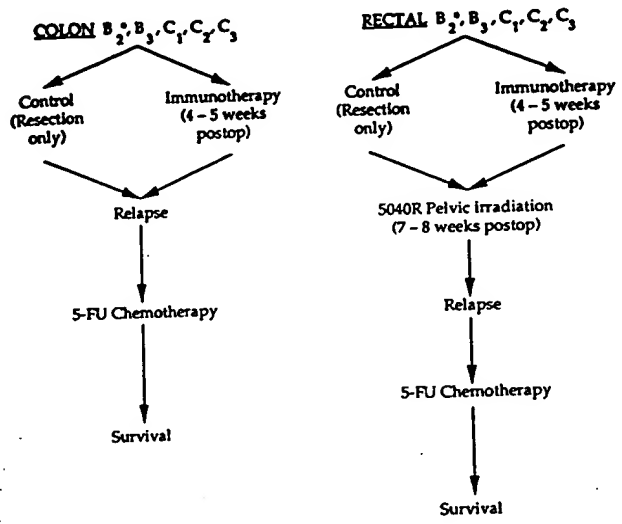

Fig 1. Schema for postoperative adjuvant ASI for colorectal cancer. Patients were grouped according to the tumor site (colon or rectal). *Each pathologic stage group was then randomized separately.

Corporation, Chicago, IL, and was stored at − 70°C.) In the third week, patients received one vaccination of 10^7 irradiated tumor cells alone. The first vaccine was placed on the left anterior thigh approximately 10 cm below the groin crease, the second in a comparable location on the right thigh, and the third in the right deltoid area. The upper thigh site was chosen because of the large lymph node basin in the immediate drainage area.

On the days of vaccination, the frozen tumor cells were thawed and the vaccines prepared according to procedures detailed previously.[15-17] The tumor cells were irradiated with 200 Gy before administration. Experience in the guinea pig model has shown that irradiation destroys the tumorogenicity, but not the immunogenicity

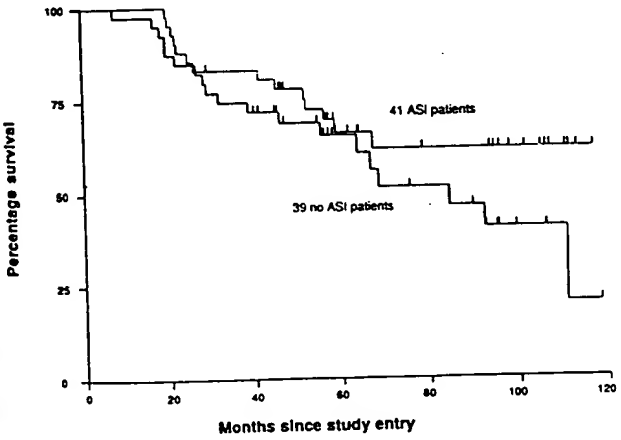

Fig 2. Kaplan-Meier curves for survival in the 80 eligible patients (colon and rectal). The hazards ratio is 1.84 in favor of ASI, but the two-sided *P* value is .088. (· · · · ·), ASI in all curves; ———, control.

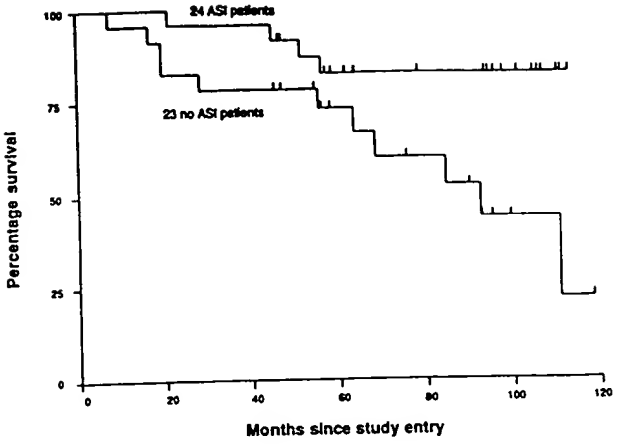

Fig 3. Distribution of months to death in 47 eligible patients with colon cancer. The hazards ratio is 3.97 in favor of ASI. The two-sided *P* value is .02.

of the cells. The BCG was added and mixed with the tumor cells just before injection.

The patients were observed closely after each vaccination for erythema and induration at the site of injections, fever, lymphadenopathy, or any adverse reactions. Since control patients did not receive a placebo injection, blinding of the study was not possible. With only objective end points in the study, blinding was not felt to be essential.

Both control and immunized patients were scheduled for monitoring at 3-month intervals for the first 2 years, every 4 to 6 months for the next 3 years, and once a year after 5 years. Complete blood cell counts, CEA, and tests of liver and renal function were to be performed at each visit. Physical examinations, including stool guaiacs, were also to be performed at each visit. Chest x-rays were scheduled at 6-month intervals, and liver scans and colonoscopy and/or air-contrast barium enemas were to be performed yearly. Most patients conformed well to the follow-up schedule. A documented histologic diagnosis by percutaneous or colonoscopic biopsy or reoperation was required to confirm recurrence of tumor, except in cases of lung or liver metastases with unequivocal x-ray or scan changes. Abnormal CEA levels alone were insufficient evidence for recurrence without histologic or other confirmation. The date of recurrence was listed as the date of the first abnormal test result that led to the definitive diagnosis of recurrence. At relapse, patients were candidates for surgical resection, systemic chemotherapy, or infusional chemotherapy depending on the extent and sites of recurrence.

Statistical Analysis

Statistical analysis used the Cox proportional hazards model in EGRET software (Statistics and Epidemiology Research Corporation, Seattle, WA). Informal examination of the empirical hazard function indicates the validity of the proportional hazards assumption. All *P* values are two-sided and are based on a Wald test of the treatment coefficient in a Cox model that adjusts for stage (B or C).[19] Figures 2 through 4 display the Kaplan-Meier estimates for the distribution of the survival and the disease-free survival.[20] Disease-free survival was defined as the time to a recurrence of any malignancy or death, censored at December 31, 1990 for living patients.

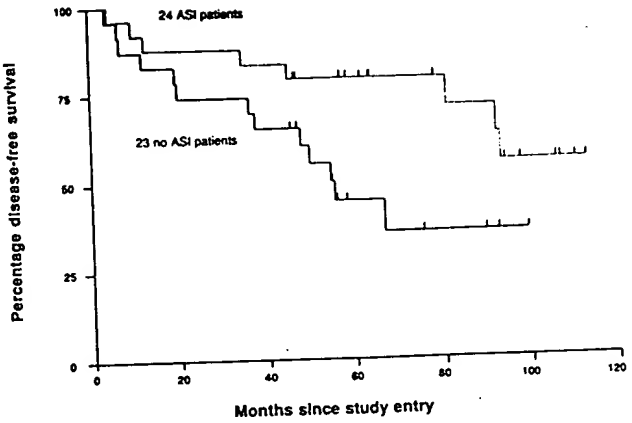

Fig 4. Distribution of months to occurrence of any malignancy or death in 47 eligible patients with colon cancer. The hazards ratio is 2.67 in favor of ASI. The two-sided *P* value is .039.

RESULTS

This report includes all patients (n = 98) who participated in the trial between March 1981 and December 1990. Every patient's record was audited by professional medical record examiners and medical oncologists and found to be accurate for all reported data using verified source documentation. Of the 98 randomized patients, the only patients not actively followed were four patients who were randomized to treatment but withdrew from the study before treatment and were lost to follow-up (Table 2). For the 94 randomized patients with complete follow-up, the median follow-up for 56 colon cancer patients is 83 months, and for 38 rectal cancer patients 57 months. For the 80 eligible patients, the median follow-up for 47 colon cancer patients is 93 months, and for 33 rectal cancer patients 58 months. No eligible patients have been lost to follow-up.

Tumor-Cell Vaccines

Tumor was dissociated and cryopreserved for 232 colorectal cancer patients viewed as potential candidates. Of these, 80 declined to participate; pathology and operative reports excluded 41 on the basis of stage (A, B_1, or D) or multiple primaries; 10 had other serious medical conditions; and technical problems with vaccine preparation excluded three patients. The remaining 98 consented to participate and were randomized.

The mean time from resection to the beginning of dissociation was 1.5 hours (range, 0.7 to 3.0), and from dissociation to cryopreservation was 5.1 hours (range, 2.5 to 9.0).

The mean weight of the tumor tissue obtained from the fresh pathology specimen was 8.3 g (range, 2.8 to 22.1). The dissociation produced a mean yield of 2.2×10^7 cells/g tumor (range, 0.5 to 10.6×10^7 cells/g). A mean prefreeze viability of 85% (range, 69% to 98%) was obtained. After thawing, the viability of the tumor cells ranged from 50% to 88%, with a mean of 77%. Within these specified viability percentages, no correlation was seen between post-thaw viability and outcome.

Patient Characteristics

Ninety-eight patients entered the trial and were randomly assigned to the immunotherapy or control groups. Four of these patients withdrew before treatment and were lost to follow-up, leaving a total of 94 patients. As listed in Table 2, 14 randomized patients have been excluded from the final statistical analysis as ineligible for the stated reasons, leaving 80 eligible patients. It should be noted that patients excluded because of advanced disease status are equally distributed between control and treatment groups (Table 2). Most of the inappropriate randomizations occurred early in the study, when patients were randomized in the immediate postoperative period of hospitalization based on the data available in the chart at that time, before the final pathology, operative, or radiology reports might show a more advanced stage. Later, patients were randomized only after all of the reports were complete. Patients were not ineligible for randomization on the basis of an elevated postoperative CEA alone, but were excluded if the CEA never returned to normal (presumed to be stage D, n = 3). We realize that excluding patients after randomization potentially detracts from the benefits conferred by randomization, especially in a small trial.[21]

The two treatment groups were remarkably similar with

Table 2. Distribution of Patients by Treatment Group

Patients	Treatment Group (no. of patients)		Total
	Treated	Control	
Enrolled (100%)	50	48	98
Excluded			
Withdrew before treatment	4	0	4
Treated in error	0	2	2
Wrong protocol (stage D)	0	1	1
Ineligible pathology (stage D)	3	3	6
Elevated (postoperative) CEA	2	1	3
Previous cancers	0	1	1
Dual primary	0	1	1
Total excluded	9	9	18
Eligible patients			80 (82%

respect to age, sex, race, location of primary tumor, pathologic stage, size of primary, extent of colon or rectal resection, and, in patients with stage C tumors, number of positive regional lymph nodes (Table 1). Five patients included as stage B_2 but with equivocal pathology findings (B_1/B_2) were equally distributed between the groups. Two were treated patients who failed to respond. Three were control patients, only one of whom failed to respond.

Protocol Violations

As explained earlier, from 1981 until 1983, patients with malignancies controlled more than 5 years earlier were considered eligible. Since 1983, patients with any prior cancer (except basal cell or squamous cell skin cancers) have been excluded. Only three patients with prior malignancies are included in the eligible study patients. None of these patients had recurrences that were related to their previous tumor. One patient had a prior early (B_1) rectal cancer that recurred in the bed of the C_3 right colon cancer for which she was entered on protocol. The second patient had a prior squamous cell carcinoma of the parotid, but died of adenocarcinoma metastatic to the lungs after entering the trial with a B_2 rectal cancer. The third patient had breast cancer treated with a mastectomy and radiation therapy 16 years earlier, but died cancer-free 5 years after entry for a C_2 colon cancer.

Protocol violations in eligible patients are listed in Table 3. These were related primarily to patients pursuing additional therapy outside of the protocol or refusing the recommended pelvic irradiation for rectal cancer. Interestingly, there have been no pelvic recurrences in the four control patients who received no pelvic irradiation or the one treated patient who received only 22 Gy. The treated patient who received four vaccines had most of the first vaccine leak from the syringe. One patient had an unusually intense reaction to her first vaccine and received no further vaccines. A second patient with an intense reaction had the BCG dose halved in the second vaccine. Two of three colon cancer patients in the ASI arm who received abdominal irradiation in violation of the protocol show no evidence of disease, while the one such patient in the control arm has recurred. Since abdominal irradiation may decrease relapse rates,[3] this imbalance of ad hoc therapy could affect the outcome in a small trial. These patients with protocol violations are all included in the analysis of eligible patients.

Immunotherapy-Related Morbidity

No serious side effects were demonstrated in the immunized patients. Since the therapy was conducted on an outpatient basis, meaningful data relative to febrile responses to the vaccine are not available. All patients were asked to keep their own temperature charts, but few recorded more than a minimal elevation of temperature in the first 2 days after receiving the first two vaccines containing BCG. As expected, all patients developed a superficial ulceration at the sites of the first and second vaccinations containing BCG. The ulcers usually occurred around the third week, were 1.5 to 2 cm in diameter, and usually healed within 3 months. Sixty percent developed palpable ipsilateral inguinal adenopathy. In each case, the adenopathy resolved within 3 months. Satellite ulcers within 5 cm of the vaccine site occurred in two patients. These resolved without any treatment. One patient had an exaggerated response to the first vaccine with induration of 15 cm and 8 cm of central ulceration. Healing occurred over a period of 3 months. She received no additional vaccines.

Liver and renal function tests were not altered by the immunotherapy. Total lymphocyte counts and absolute lymphocyte counts did not change significantly.

Skin Test Reactivity

Extensive skin test data on immunized and control patients have been published.[16] Our published data on 24

Table 3. Protocol Violations

Site/Stage	Violation	Current Status
Treated patients		
Colon/C_2	Abdominal RT	NED
Colon/C_2	Abdominal RT & chemotherapy	NED
Colon/C_2	Abdominal RT	Alive with hematologic malignancy
Colon/C_2	4 vaccines	Died of metastasis
Colon/B_2	1 vaccine only	NED
Colon/B_2	½ dose BCG in second vaccine	NED
Rectal/C_1	22 Gy of RT	Died of pulmonary metastasis
Rectal/C_2	RT started 8 days early	NED
Control patients		
Colon/B_2	Abdominal RT	Alive with new primary
Rectal/B_2	Refused postoperative RT	Died of pulmonary metastasis
Rectal/C_2	Chemotherapy	NED
Rectal/C_2	No RT	Died of pulmonary metastasis
Rectal/B_2	Refused postoperative RT	NED
Rectal/C_2	Refused postoperative RT	NED
Rectal/C_2	Refused postoperative RT	Alive with pulmonary metastasis

Abbreviations: RT, radiation therapy; NED, no evidence of disease.

immunized and 11 control patients with colorectal cancer include some patients from a stage D protocol. All patients reacted initially to at least one of the standard recall antigens. There was no change in recall antigen reactivity in the follow-up period, except that all but one of the immunized patients converted to PPD-positive. The DCH response to autologous tumor cells compared with normal mucosa cells was increased significantly ($P < .01$) in that 67% of the immunized patients showed a positive response, but only 9% of nonimmunized patients tested showed positive reactivity at the comparable postoperative period. There was not a similar boost to autologous mucosa cells in the immunized patients. In the adjuvant trial now being reported, 20 of the 41 immunized patients were tested preimmunization and postimmunization for DCH with autologous tumor cells and mucosa. Of these 20 patients, 16 (80%) became positive: 86% of colon cancer patients and 67% of rectal cancer patients.

Clinical Outcome

In our prior interim report of this trial,[17] colon and rectal carcinoma patients were analyzed together because of the small number of patients. In this report, we analyzed the total population and, recognizing the postimmunotherapy treatment difference for rectal cancer patients, performed a cohort analysis for colon and rectal carcinoma.

We considered two outcomes: survival and disease-free survival. In contrast to the 1985 interim analysis, the Cox model analysis provides no evidence of a treatment by stage interaction to support such a stratification by stage. Furthermore, the study is not powerful enough either to test the hypothesis of interaction or to compare treatment outcomes for patients separately within stages. Therefore, although the randomization was performed by stratification by stage, as well as site, the statistical decision was to view stage as dichotomous.

Table 4 lists the survival data for the 94 randomized patients (colon and rectal, eligible and ineligible, with complete follow-up), as well as for the 80 eligible patients. All analyses were adjusted for stage. Among the 94 randomized patients with complete follow-up, 46 received ASI and 48 were in the control group. Deaths from all causes were included in this analysis. In the total population of patients randomized to ASI compared with the control group, the positive trend reported in 1985[17] continues, but while the difference is impressive, it is no longer significant ($P = .068$). In the treated colon cancer patients, there was a significant survival advantage compared with controls ($P = .026$), with a hazards ratio of 2.83.

Among the 80 eligible patients, 41 received ASI and 39 were in the control group. Deaths from any cause were included in this analysis. Again, an overall positive trend is observed ($P = .088$), but the only significant survival difference ($P = .02$) was seen in the comparison of the ASI-treated colon cancer patients with the controls. The hazards ratio was 3.97.

Figures 2 and 3 show the distribution (using Kaplan-Meier[20] estimates) of survival. The Cox regression model[19] was used to compare treatment groups. Figures 2 and 3 depict the survival, respectively, for the 80 eligible patients combining colon and rectal patients and the 47 colon cancer patients.

Disease-free survival rates among the 80 eligible patients are summarized in Table 5. These analyses are expressed as disease-free survival using recurrence of any malignancy or death. This conservative analysis is of interest, as we do not know if this approach to ASI is specific for colorectal cancer or might confer broader resistance to other malignancies. When the occurrence of any malignancy or death was evaluated, there was a significant difference among the colon cancer patients ($P = .039$; hazards ratio. 2.67) (Fig 4). When the recurrence event included colon tumors only, there was a statistically significant difference between the treated and control colon cancer patients ($P = .03$; hazards ratio, 2.97).

Table 4. Summary of Survival

Patient Group	Tumor	ASI Treatment		Control		P	Hazards Ratio* (adjusted for stage)	Confidence Limits
		Deaths/Total	%	Deaths/Total	%			
Randomized (n = 94)	All	19/46	41.3	26/48	54.2	.068	1.75	—
	Colon	7/27	25.9	15/29	51.7	.026	2.83	1.13, 7.07
	Rectal	12/19	63.2	11/19	57.9	.772	1.13	—
Assessable (n = 80)	All	14/41	34.1	19/39	48.7	.088	1.84	—
	Colon	4/24	16.7	11/23	47.8	.020	3.97	1.24, 12.72
	Rectal	10/17	58.8	8/16	50.0	.930	0.95	—

*A hazards ratio > 1 favors the treatment group.

Table 5. Summary of Disease-Free Survival in 80 Eligible Patients

Type of Recurrence	Tumor	ASI Treatment		Control				
		Recurrence or Deaths/ Total	%	Recurrence or Deaths/ Total	%	P	Hazards Ratio* (adjusted for stage)	Confidence Limits
Any malignancy or death	All	19/41	46.3	23/39	58.9	.147	1.58	—
	Colon	8/24	33.3	13/23	56.5	.039	2.67	1.05, 6.76
	Rectal	11/17	64.7	10/16	62.5	.905	1.05	—
Primary or second colon tumor or death	All	17/41	41.5	23/39	58.9	.112	1.67	—
	Colon	6/24	25.0	13/23	56.5	.030	2.97	1.11, 7.94
	Rectal	11/17	64.7	10/16	62.5	.905	1.05	—

*A hazards ratio > 1 favors the treatment group.

In Figs 3 and 4, it is apparent that late events (> 60 months) are important in survival and disease-free survival. The three late events in the treated group involve diagnoses of malignancies unrelated to the primary tumor (a second primary colon cancer and two hematologic malignancies). In the control group, three of four late events were due to progression of the primary tumor and one was death due to a new primary tumor (renal cancer).

DISCUSSION

This report updates our previously published, prospectively randomized, controlled trial of ASI in colon and rectal cancer.[17] A statistical analysis of this small trial suggests therapeutic benefit from autologous immunization in patients with colon but not rectal cancer, although with such small numbers of patients we cannot conclude that ASI is of proven therapeutic benefit.

That colon cancer patients appear to benefit while rectal cancer patients do not is of considerable interest. It could be that rectal cancers are intrinsically less immunogenic. We do not have sufficient data to confirm that hypothesis on the basis of DCH reactivity. We are concerned that the lack of ASI effect in rectal cancer patients could be related to the logistics of the pelvic irradiation. In the guinea pig model, the draining lymph nodes must be left intact for 21 days following immunization to develop sufficient immunity to destroy tumor. Rectal cancer patients started pelvic irradiation, including the draining lymph nodes, within a few days after completing immunization. It is possible that the lymph nodes targeted by immunotherapy were destroyed by the irradiation before they could have a full impact on the immunologic response.

We now have three lines of evidence that ASI has an immunologic impact in patients with colon cancer: (1) we have previously reported a significant boost in reactivity (48-hour DCH) to autologous tumor cells in vaccinated patients[16]; (2) we have reported the use of peripheral-blood lymphocytes from these immunized patients as sources for the development of stable clones of human B lymphocytes that produce colon and rectal tumor-specific monoclonal antibodies[22] (lymphocytes harvested from the peripheral blood of unimmunized patients are less likely to contain cells that produce tumor-specific monoclonal antibodies); and (3) the sustained differences in clinical outcome between immunized and nonimmunized groups of patients with colon cancer in our study are encouraging, even with the small numbers.

How can we explain our modest gains in the face of so many past failures of attempts to control malignant disease by immune manipulations? A reappraisal of the past failures in the light of current studies in experimental animal models may clarify the reason for the failures and suggest other new immunologic approaches. Our clinical protocol emerged from biologic principles derived from nearly 8 years of investigation with the guinea pig line-10 hepatocarcinoma model. Studies in the guinea pig model demonstrated that BCG mixed with tumor cells is effective in inducing a degree of systemic tumor immunity capable of eliminating a limited disseminated tumor burden. Further studies demonstrated the feasibility of preparing tumor-cell suspensions from enzymatically dissociated solid tumors without loss of immunogenicity, a requirement for the preparation of human tumor vaccines.[14] It was possible in this model to demonstrate that allogeneic cells, dead cells, or cell components (antigen extracts) were not effective. It also was demonstrated that BCG components were not effective substitutes for whole viable BCG cells. Autologous tumor cells alone or BCG alone were not effective.

Past attempts at controlling colon cancer by immunologic means have used primarily nonspecific immunomodulators, none of which have significantly altered the course of the disease. Interestingly, the National Surgical Adjuvant Breast and Bowel Project protocol C-01 reported a survival advantage in favor of BCG scarification

treatment over resection alone in patients with Dukes' stage B and C carcinoma of the colon.[23] However, further investigation disclosed that the survival advantage in favor of BCG was a result of a diminution in non–cancer-related deaths. Deaths from cardiovascular-related sequelae were twice as common in the untreated control group as in the BCG-treated cohort. In the present study, there were two non–cancer-related deaths, one in the control colon cancer group and one in the ASI-treated colon cancer group.

Most human trials with tumor vaccines have had at least one major variation from the requirements listed above for successful immune stimulation with ASI. Most have used tumor cells of low viability, an absolute predictor of failure in our model. Such cells disintegrate rapidly and do not provide the necessary sustained antigenic stimulus to the regional lymph nodes. Others have used allogeneic cell lines. Unless cross-reacting tumor antigens are shared by the donor and recipient, allogeneic vaccines are destined to fail.

In the guinea pig model, the success of therapy is inversely proportional to the number, size, and location of metastases present at the time of treatment. It is unlikely that even the most optimal vaccination regimen could control large tumor burdens. Studies in the guinea pig model have suggested that ASI can become a meaningful, integral part of combination therapy, when coupled with conventional chemotherapy or additional biologic therapy.[24] Studies have shown that chemotherapy acts synergistically with ASI in the eradication of pulmonary micrometastases if a precise sequence of immunotherapy and chemotherapy is followed.[25,26] The mechanism for this synergistic interaction appears to be an immune-mediated breakdown of the normal anatomic and vascular barriers in solid tumors, allowing greater access of blood-borne chemotherapeutic agents to the tumor cells.

We are attempting to verify the potential efficacy of ASI therapy through prospectively randomized studies underway under the auspices of the ECOG using ASI in an adjuvant setting in Dukes' stage B and C colon cancer patients. The Dukes' stage C study evaluating ASI alone has been closed with adequate accrual for reliable assessment and accrual continues with the Dukes' stage B colon patients. A second phase III trial has been initiated combining immunotherapy and chemotherapy in patients with advanced disease. With the recently reported benefits from adjuvant 5-FU and levamisole,[6,7] our adjuvant protocols have been altered to include 5-FU and levamisole in all Dukes' stage C colon cancer patients. This will give an optimal opportunity to determine the efficacy of ASI in combination with chemotherapy in the adjuvant setting.

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10. Hanna MG Jr, Peters LC: Specific immunotherap. · established visceral micrometastases by BCG-tumor cell vaccine alone or as an adjunct to surgery. Cancer 42:2613-2625, 1978

11. Hanna MG Jr, Pollack VA, Peters LC, et al: Active-specific immunotherapy of established micrometastases with BCG plus tumor cell vaccines: Effective treatment of BCG side effects with isoniazid. Cancer 49:659-664, 1982

12. Hoover HC Jr, Peters LC, Brandhorst JS, et al: Therapy of spontaneous metastases with an autologous tumor vaccine in a guinea pig model. J Surg Res 30:409-415, 1981

13. Key ME, Hanna MG Jr: Mechanism of action of BCG-tumor cell vaccines in the generation of systemic tumor immunity: 1. Synergism between BCG and line 10 tumor cells in the induction of an inflammatory response. J Natl Cancer Inst 67:853-861, 1981

14. Peters LC, Brandhorst JS, Hanna MG Jr: Preparation of immunotherapeutic autologous tumor cell vaccines from solid tumors. Cancer Res 39:1353-1360, 1979

15. Peters LC, Hanna MG Jr: Active-specific immunotherapy of established micrometastasis: Effect of cryopreservation procedures on tumor cell immunogenicity in guinea pigs. J Natl Cancer Inst 64: 1521-1525, 1980

16. Hoover HC Jr, Surdyke M, Dangel R, et al: Delayed cutaneous hypersensitivity to autologous tumor cells in colorectal cancer patients immunized with an autologous tumor cell: Bacillus Calmette-Guérin vaccine. Cancer Res 44:1671-1676, 1984

17. Hoover HC, Surdyke MG, Dangel RB, et al: Prospectively randomized trial of adjuvant active-specific immunotherapy for human colorectal cancer. Cancer 55:1236-1243, 1985

18. Gunderson LL, Sosin H: Areas of failure found at reoperation following "curative surgery" for adenocarcinoma of the rectum: Clinicopathologic correlation and implications for adjuvant therapy. Cancer 34:1278-1292, 1974

19. Cox DR: Regression models and life tables (with discussion). J R Stat Soc B 34:187-220, 1972

20. Mantel N: Evaluation of survival data and two new rank order statistics arising in its consideration. Cancer Chemother Rep 50:163-170, 1966

21. Armitage P: Exclusions, losses to follow-up, and withdrawals in clinical trials, in Shapiro SH, Lous TA (eds): Clinical Trials, Issues and Approaches. New York, NY, Dekker, 1988, pp 99-113

22. Haspel MV, McCabe RP, Pomato N, et al: Generation of tumor cell-reactive human monoclonal antibodies using peripheral blood lymphocytes from actively immunized colorectal carcinoma patients. Cancer Res 45:3951-3961, 1985

23. Wolmark N, Fisher B, Rockette H, et al: Postoperative adjuvant chemotherapy or BCG for colon cancer: Results from NSABP Protocol C-01. J Natl Cancer Inst 80:30-36, 1988

24. Berd W, Maguire HC, Mastrangelo MJ: Induction of cell-mediated immunity to autologous melanoma cells and regression of metastases after treatment with a melanoma cell vaccine preceded by cyclophosphamide. Cancer Res 46:2572-2577, 1986

25. Hanna MG Jr, Key ME: Immunotherapy of metastases enhances subsequent chemotherapy. Science 217:367-369, 1982

26. Key ME, Brandhorst JS, Hanna MG Jr: Synergistic effects of active specific immunotherapy and chemotherapy in guinea pigs with disseminated cancer. J Immunol 130:2987-2992, 1983

Exhibit A

CURRICULUM VITAE

Donald P. Braun, Ph.D.

January, 2000

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HOME ADDRESS: 14954 Stonehaven Dr.
Perrysburg, Ohio, 43551

PERSONAL: Born: New York, NY; March 7, 1950
SS # 355-44-2224
Married: Judy Braun
Children: Jennifer, Matthew, Bethany

EDUCATION AND TRAINING:

1972	University of Illinois, Urbana, IL, B.S. degree.
1974	University of Illinois at the Medical Center, Chicago, IL, M.S. degree.
1976	University of Illinois at the Medical Center, Chicago, IL, Ph.D. degree.

CHRONOLOGY OF EMPLOYMENT:

1976-1977	Research Associate, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL.
1977-1978	Instructor, Department of Microbiology, University of Illinois at the Medical Center, Chicago, IL
1978-1979	Research Associate, Section of Medical Oncology, Rush-Presbyterian-St. Luke's Medical Center, Chicago, IL.
1979-1980	Instructor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1981-1983	Assistant Professor, Department of Medicine; Assistant Professor, Department of Immunology/ Microbiology, Rush Medical College, Chicago, IL.
1983-1987	Associate Professor, Department of Medicine; Assistant Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1987	Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.

1989	Associate Director, Section of Medical Oncology (for Research); Associate Professor, Department of Medicine; Associate Professor, Department of Immunology/Microbiology, Rush Medical College, Chicago, IL.
1993-1999	Director, Scientific Program Development. Rush Cancer Institute.
1993-1999	Professor of Medicine and Immunology/Microbiology.
1999-present	Administrative Director of the Cancer Institute, Medical College of Ohio
1999-present	Professor, Department of Surgery, Medical College of Ohio.

FEDERAL GOVERNMENT/PUBLIC ADVISORY COMMITTEES:

1982-1984	Member, Experimental Therapeutics Study Section, National Cancer Institute.
1983,1985	Member, Small Business Innovation Grant Review Study Section, NCI.
1985-1988	Member, Experimental Therapeutics I Study Section, NCI.
1985	Chairman, Experimental Therapeutics Special Study Section, NCI.
1985-1992	Biological Response Modifier Committee, Illinois Cancer Council
1986-present	Reviewing Member, Arizona Disease Control Research Commission.
1988-1989	Member, Chicago Leukemia Research Society.
1988-1989	Member, Small Business Innovation Grant Review Study Section, NCI.
1990, 1993	Ad Hoc reviewer, Experimental Therapeutics Study Section 1, NCI.
1991, 1992	Ad Hoc reviewer, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
1994-1998	Member, Immunology and Immunotherapy Study Section, American Cancer Society-National Division.
1999-present	Advisory Member, "Molecular Targets for therapy of Lung Cancer", National Cancer Institute/CTEP.
1999-present	Advisory Member, Ohio Cancer Incidence Surveillance System
2000	American Cancer Society Immunology and Immunotherapy Study Section, National Division-ad hoc review.

CONSULTANT POSITIONS:

Burrough's Wellcome, 1983-1984
Pfizer Pharmaceutical, 1986-1988
Boehringer Mannheim, 1991-1993
Abbott Laboratories, 1993
Institute for the Study and Treatment of Endometriosis, 1990-present
Adeza Biomedical, 1993
Imutec Corporation, 1993-present
Imutec Corporation; Chairman, Medical Advisory Board, July, 1995-present.
RxKinetic Inc.; Chairman, Medical Scientific Advisory Board. 1997-present.

COMPETITIVE EXTRAMURAL GRANT AWARDS: (note: as Principal Investigator or co-Principal Investigator only)

1. "Cancer Drug Effects on Patient Suppressor Cells". Source: NIH/NCI # CA27598 Period of Support: 09/01/80-03/31/87; as co-Principal Investigator.

2. "Immune Testing in Lung CA During Specific Immunotherapy". Source: NIH/NCI # CA26138. Period of Support: 07/01/80-06/30/83; as co-Principal Investigator.

3. "A Phase I Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2). Source: NIH/NCI # RFA No-1-CM47667-BRM-MA01. Period of Support: 09/30/84-03/31/87. Subcontract from the Illinois Cancer Council; as Laboratory Principal Investigator for Rush Component.

4. "Phase IB and/or Phase II Clinical Trial of Natural and Recombinant Interleukin-2 (IL-2). Source: NIH/NCI # RFA No-1 CM47667-03 BRM-MA-04. Period of Support: 09/29/85-02/28/89. Laboratory Principal Investigator for Rush Component.

5. "Arachidonate Metabolism in Cancer Patient Macrophages". Source: NIH/NCI # CA41741.Period of Support: 07/01/88-06/30/92; as Principal Investigator.

6. "LAK Function in Tumor-Infiltrating Leukocytes of Cancer Patients". Source: American Society of Clinical Oncology- 1990 Young Investigator Award to E. Staren, M.D.; as Mentor.

7. "Cancer Patient Macrophage Function in Tumor Environments". Source: NIH/NCI #CA58922. Period of Support: 12/31/92-07/01/96 as Principal Investigator.

8. "American Cancer Society New Investigator Grants in Cancer Research". Source: American Cancer Society. Period of Support: 06/31/95-07/01/97 as Principal Investigator.

9. "Cancer Drug Modulation of Tumor Sensitivity to Macrophages". Source: NIH/NCI, period of support: 12/01/00-11/30/05, as Principal Investigator. status-pending.

10. "Cyclooxygenase Metabolism in Cancer Patient Psychoneuroimmunology". Source: American Cancer Society. Period of Support: 01/01/01-12/31/06, as Principal Investigator. Status-pending.

NONCOMPETITIVE EXTRAMURAL FUNDING: (as principal or co-principal investigator)

1. "The Effect of CGS13080, CGS14854 and CGS53913 on Arachidonic Acid Metabolites and Immune Status of Patients with Solid Tumors". Source: Ciba-Geigy. Period of Support: 10/01/87-09/30/88; as Principal Investigator.

2. "Phase III Protocol for Evaluation of Combined Modalities in the Treatment of Colonic Carcinoma with Positive Nodes, Duke's C, Surgical Resection Alone vs. Postoperative Immunotherapy followed by Chemotherapy". Source: Litton Institutes. Period of Support:06/30/88-present; as Laboratory Principal Investigator for Rush.

3. "Immunologic Testing and Limited Feldene Administration to Patients with Upper Aerodigestive Tract Squamous Cancer". Source: Pfiser Laboratories, Pfiser Inc. Period of Support: 10/01/89-06/30/91; as Principal Investigator.

4. "Macrophage Function in Women with Endometriosis". Source: Sterling International. Period of Support: 04/01/90-10/01/91; as Principal Investigator for Rush component.

5. "Macrophage Regulation of Endometrial Cell Growth in Women with Endometriosis". Source: Sterling International. Period of Support: 03/01/92-10/01/93; as Principal Investigator for Rush component.

6. "Mechanisms for Modulation of Macrophage Tumoricidal Function in Cancer Patients by Virulizin". Source: Imutec Corporation. Period of Support: 02/01/94-12/01/95; as Principal Investigator.

7. "Immunological Modulation in Pancreatic Cancer Patients treated with Virulizin". Source: Imutec International. Period of Support: 06/30/96-12/31/98; as Principal Investigator.

8. "Modulation of Macrophage Cytolytic Function by Virulizin in Endometriosis". Source: Imutec International. Period of Support: 06/30/96-12/31/97; as Principal Investigator.

9. "Interaction of HIP/PCA particles with leukocytes from Cancer Patients". Source: RxKinetix. Period of Support: 01/01/98 - 08/31/2001; as Principal Investigator.

10. "Endothelin Regulation of Tumor Proliferation and Apoptosis in Human Intracranial Malignancy". Source: Abott Laboratories. Period of Support (pending-to begin in 2000); as Principal Investigator.

11. "Amelioration of TNFα effects in endometriosis by Enbrel". Source: Immunex Corp. Period of support: 01/01/00-06/30/01. As Laboratory Principal Investigator.

11. "H11 binding to human cancer cells". Source: Novopharm Biotechnology. Period of Support: 05/01/00-09/01/01. As Principal Investigator.

HONORS:

USPHS Immunology Trainee, 1973-1974.
USPHA Oncology Trainee, 1974-1976.
Milan V. Novak Award, University of Illinois, Department of Microbiology, 1977.
Who's Who in Cancer Research, 1985
American Men and Women in Science, 1988
1st place award for original research, American Fertility Society, 1992.
Chairman, Poster-Discussion Session, AACR, 1994.
Chairman, Immunology Plenary Session, Vth International Conf. on Endometriosis, 1996.
Chairman, Rationale for Immunotherapy in Endometriosis: VI World Congress on Endometriosis, Quebec City, Canada, 1998.

ACTIVE MEMBERSHIPS:

American Association for Cancer Research
American Chemical Society
American Association for the Advancement of Science
New York Academy of Science
Society of Biology Response Modifiers
American Fertility Society
American Society of Reproductive Medicine

PUBLICATIONS:

BOOKS EDITED:

1. Prostaglandin Inhibitors in Cancer Immunology and Immunotherapy. eds. JE Harris, DP Braun and KM Anderson. CRC Press, Boca Raton, FLA, 1994.

REVIEWS AND BOOK CHAPTERS:

1. Dray S, and Braun DP: Some perspectives on the transfer of cell mediated immunity by immune RNA. Mol Cell Biochem 25:15, 1979.

2. Braun DP, and Harris JE: Serial immune function testing to predict clinical disease relapse in patients with solid tumors. Cancer Immunol Immunother 15:165, 1983.

3. Harris JE, and Braun DP: The effect of cytotoxic drugs on immunoregulatory cell function in solid tumor cancer patients. Clin Immunol Newsletter 5:113-116, 1984.

4. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. In: Proceedings of the 3rd International Symposium of the Evaluation of the Immunomodifiers, 1984.

5. Braun DP, and Harris JE: Modulation of the immune response by chemotherapy. In: The Modulation of Immunity. Mitchell MS (ed), Oxford: Pergamon Press, 1985.

6. Braun DP, and Harris JE: Effects of cytotoxic chemotherapy on immune function in cancer patients. Cancer Treat Symp 1:19-26, 1985.

7. Braun DP, and Harris JE: Cancer chemotherapy and its impact on the immune system. In: Fundamentals of Cancer Chemotherapy. Carter SK, and Hellman K (eds), New York: McGraw-Hill, pp 77-97, 1986.

8. Von Roenn J, Harris JE, and Braun DP: Suppressor cell function in solid tumor cancer patients. J Clin Oncol 5:150-159, 1987.

9. Dmowski WP, Braun DP and Gebel H: Endometriosis: Genetic and Immunologic Aspects. in: Current Concepts in Endometriosis. 2nd International Symposium on Endometriosis. Alan R. Liss, Inc. New York, p 99-122, 1989.

10. Dmowski WP, Braun DP and Gebel H: The Immune System in Endometriosis. in Modern Approaches to Endometriosis. J. Rock ed. Kluwer Academic Publishers, p 97-111, 1991.

11. Braun DP and Groenwald SL: The Immune System and Cancer. in Cancer Nursing: Principles and Practice. third edition. Groenwald SL and Goodman M eds. Jones and Bartlett, Boston, MA, pp 70-85, 1993.

12. Harris, J.E. and Braun, D.P.: Tumor Vaccination. in Cancer: Concept to Clinic. Medical Publishing Enterprises. Fair Lawn, NJ; E. Borden, ed. pp. 28-31, 1993.

13. Braun DP: The Impact of Prostaglandins on Cancer Patient Immunity. in Prostaglandin Inhibitors in Tumor Immunology and Immunotherapy. Harris JE, Braun DP and Anderson KM, eds. CRC Press, Boca Raton, Florida, pp. 109-129, 1994.

14. Dmowski WP, Gebel HM and Braun DP. The Role of Cell-Mediated Immunity in Pathogenesis of Endometriosis. Acta Obstet. Gynecol. Scand. Suppl. 73:7-14, 1994.

15. Dmowski WP and Braun DP. Immunological Aspects of Endometriosis. Contemp. Rev. Obstet. Gynaecol. 7: 167-171, 1995.

16. Dmowski WP, Braun DP, and Rotman, C. Aspectos Immunologicos de la endometriosis. in Reproducion Humana. Remonhi, J, Simon C, Pellicer, A and Bonilla-Musoles, eds. McGraw Hill InterAmericana, Madrid, pp 195-204, 1996.

17. Braun DP and Dmowski WP. Endometriosis: Abnormal Endometrium and Dysfunctional Immune Response. Current Opinion in Obsterics and Gynecology. 10:365-369, 1998.

18. Dmowski WP, Gebel H, and Braun DP. Decreased Apoptosis and sensitivity to macrophage-mediated cytolosis of endometrial cells in endometriosis. Human Reproduction. In Press, 1999.

ARTICLES:

1. Braun DP, and Dray S: Immune RNA mediated transfer of tumor antigen responsiveness to unresponsive peritoneal exudate cells from tumor bearing animals. Cancer Res 37:4138-4144, 1977.

2. Mortensen RF, Braun DP, and Gewurz H: Effects of C-reactive protein on lymphocyte function. III. Inhibition of antigen-induced lymphocyte stimulation and lymphokine production. Cell Immunol 28:59-68, 1977.

3. Braun DP, Hengst J, Moykr M, and Dray S: Antitumor immunity in strain 2 guinea pigs immunized with KCl extracts of L_2C tumor cells. J Natl Cancer Inst 60:899-903, 1978.

4. Mokyr M, Braun DP, Usher D, Reiter H, and Dray S: The development of *in vitro* and *in vivo* antitumor cytotoxicity in noncytotoxic, MOPC-315, tumor cells. Cancer Immunol Immunother 4:143-150, 1978.

5. Braun DP, Mokyr M, and Dray S: Generation of anti-MOPC-315 cytotoxicity in uneducated or *in vitro* educated spleen cells from normal or MOPC-315 tumor bearing mice pretreated *in vivo* with BCG. Cancer Res 38:1626-1631, 1978.

6. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in spleen cells of MOPC-315 tumor bearers. In: Cancer Immunology: Experimental and Clinical. Crispin RG (ed), p 211, 1978.

7. Mokyr M, Braun DP, and Dray S: Augmentation of antitumor cytotoxicity in MOPC-315 tumor bearer spleen cells by depletion of glass adherent cells prior to *in vitro* education. Cancer Res 39:785, 1979.

8. Mokyr M, Bennett JA, Braun DP, Hengst JCD, Mitchell MS, and Dray S: Opposite effects of different strains or batches of the same strain of BCG on the *in vitro*

generation of syngeneic and allogeneic antitumor cytotoxicity. J Natl Cancer Inst 64:339, 1980.

9. Cobleigh MA, Braun DP, and Harris JE: Age dependent changes in human peripheral blood B cell and T cell subsets: Correlation with mitogen responsiveness. Clin Immunol Immunopathol 15:162, 1980.

10. Braun DP, Cobleigh MA, and Harris JE: Selective effect of cytotoxic chemotherapy on immunoregulatory suppressor cells in solid tumor cancer patients. In: Tumor Progression. Crispen RG (ed), Philadelphia: Franklin Institute Press, pp 91-102, 1980.

11. Braun DP, Cobleigh MA, and Harris JE: Multiple concurrent immunoregulatory defects in cancer patients whose peripheral blood leukocytes exhibit depressed PHA induced lympho blastogenesis. Clin Immunol Immunopathol 17:89, 1980.

12. Cobleigh MA, Braun DP, and Harris JE: Quantitation of lymphocytes and T cell subsets (T_G and T_M cells) in disseminated solid tumor cancer patients. J Natl Cancer Inst 64:1041, 1980.

13. Paque RE, Braun DP, and Dray S: Characterization of lymphoid cell RNA which modulates specific cellular immunity. In: Second International Symposium on RNA in Development. Academia Sinics, 1980.

14. Braun DP, and Harris JE: Modulation of immune response by chemotherapy. Pharmacol Ther 14:89-122, 1981.

15. Braun DP, and Harris JE: Effects of combination chemotherapy on immunoregulatory cells in the peripheral blood of solid tumor cancer patients: Correlation with rebound-overshoot immune function recovery. Clin Immunol Immunopathol 20:143, 1981.

16. Braun DP, and Harris JE: Relationship of leukocyte numbers, immunoregulatory cell function and phytohemagglutinin responsiveness in cancer patients. J Natl Cancer Inst 67:809, 1981.

17. Braun DP, Harris JE, Maximovich S, Marder R, and Lint TF: Chemiluminescence in peripheral blood mononuclear cells of solid tumor cancer patients. Cancer Immunol Immunother 12:31, 1981.

18. Braun DP, Penn RD, Flannery AM, and Harris JE: Immunoregulatory cell function in peripheral blood of glioblastoma multiforme patients. Neurosurg 12:31, 1981.

19. DeBoer KP, Braun DP, and Harris JE: Natural cytotoxicity and antibody dependent cytotoxicity in solid tumor cancer patients: Regulation by adherent cells. Clin Immunol Immunopathol 23:133, 1982.

20. Harris JE, DeBoer KP, Vahey AL, and Braun DP: The measurement of leukocyte subsets in the peripheral blood of solid tumor cancer patients using monoclonal antibody reagents. Med Pediatr Oncol 10:185, 1982.

21. Taylor SG IV, Saffold P, Braun DP, and Harris JE: T_G cell involvement in the leukocyte adherence inhibition phenomenon. J Natl Cancer Inst 68:549, 1982.

22. Braun DP, DeBoer KP, and Harris JE: Chemiluminescence, suppression and cytotoxicity in peripheral blood mononuclear cells from solid tumor cancer patients. Cancer Immunol Immunother 14:86, 1982.

23. Harris JE, Harris ZL, and Braun DP: Effect of interferon-α on indomethacin sensitive immunoregulatory cells. In: Proceedings of the 15th International Leukocyte Culture Conference. Parker JW, and O'Brien R (eds), Chichester, England: John Wiley and Sons, Ltd., p 635, 1983.

24. Chiu K, Harris JE, Kroin J, Slayton R, and Braun DP: The immunological response of Wistar rats to intracranially implanted C-6 glioma cell line. J Neurooncol 1:365-372, 1983.

25. Penn RD, Kroin JS, Harris JE, Chiu K, and Braun DP: Chronic intratumoral chemotherapy of a rat brain tumor with cisplatin and flourouracil. Appl Neurophysiol 46:240-244, 1983.

26. Braun DP, Nisius S, Hollinshead AC, and Harris JE: Serial immune testing in surgically resected lung cancer patients. Cancer Immunol Immunother 15:114-121, 1983.

27. Braun DP, Harris ZL, Harris JE, Sandler S, Khandekar J, Locker G, Haid M, Gordon L, Shaw J, Cobleigh MA, and Gallagher P: The effect of interferon therapy on indomethacin sensitive immunoregulation in the peripheral blood mononuclear cells of renal cell carcinoma patients. J Biol Response Mod 2(3):251-262, 1983.

28. Van Epps SF, Stewart LD, Pandey JP, Fudenberg HH, Harris JE, and Braun DP: Immuno-globulin G heavy chain (Gm) allotypes in lung cancer. N Engl J Med 1983.

29. Braun DP, Harris JE, and Rubenstein M: Relationship of arachidonic acid metabolism to indomethacin sensitive immunoregulatory function and lymphocyte PGE sensitivity

in peripheral blood mononuclear cells of disseminated solid tumor cancer patients. J Immunopharmacol 6(3):227-236, 1984.

30. Braun DP, Penn RD, and Harris JE: Regulation of natural killer function by glass adherent cells in patients with primary intracranial malignancies. Neurosurg 15(1):29-33, 1984.

31. Braun DP, and Harris JE: Effects of combination chemotherapy on PGE mediated immunoregulation in the peripheral blood mononuclear cells of solid tumor cancer patients. J Biol Response Mod 3:391-396, 1984.

32. Braun DP, and Harris JE: Abnormal indomethacin sensitive suppression in peripheral blood mononuclear cells of cancer patients restricts augmentation by interleukin 2. J Biol Response Mod 3:533-540, 1984.

33. Chiu KM, McPherson LH, Harris JE, and Braun DP: The separation of cytotoxic human peripheral blood monocytes into high and low phagocytic subsets by centrifugal elutriation. J Leukocyte Biol 36:729-737, 1984.

34. Braun DP, and Harris JE: Effect of cytotoxic antineoplastic chemotherapy on immuno-regulatory leukocytes measured with monoclonal antibodies. Clin Immuno Immunopathol 33(1);54-56, 1984.

35. Leslie WT, Templeton A, and Braun DP: Kaposi's sarcoma in the acquired immune deficiency syndrome. Med Pediatr Oncol 12:336-342, 1984.

36. Braun DP, and Harris JE: Effect of chemotherapy on NK function in the peripheral blood of cancer patients. Cancer Immunol Immunother 21:240-245, 1986.

37. Braun DP, and Harris JE: Abnormal monocyte function in patients with Kaposi's sarcoma. Cancer 57:1501-1506, 1986.

38. Janus TJ, Braun DP, and Harris JE: Modulation of lymphocyte responsiveness to phyto-hemagglutinin by micromolecular fibrinogen degradation products. Clin Immunol Immuno-pathol 41:26-34, 1986.

39. Potempa LA, Gewurz H, Harris JE, and Braun DP: Stimulatory effects of the C-reactive protein subunit on monocyte function, including release of IL 1. Protides Biol Fluids 34:287-290, 1986.

40. Braun DP, Bonomi PD, Taylor SG IV, and Harris JE: Modification of the effects of cytotoxic chemotherapy on the immune responses of cancer patients with a nonsteroidal anti-inflammatory drug, piroxicam. J Biol Response Mod 6:331-345, 1987.

41. Harris JE, and Braun DP: Modulation of immune function in cancer patients. Clin Physiol Biochem 5:222-226, 1987.

42. Wiederhold MD, Ondrey F, Anderson KM, Braun DP, and Harris JE: Synthesis of eicosanoids by γ-interferon differentiated U937 cells. Clin Physiol Biochem 6:21-28, 1988.

43. McMannis JD, Fisher RI, Creekmore SP, Braun DP, Harris JE, and Ellis TM: *In vivo* effects of recombinant IL 1. I. Isolation of circulating Leu 19^+ lymphokine activated killer effector cells from cancer patients receiving recombinant IL 2. J Immunol 140:1335-1340, 1988.

44. Harris JE, and Braun DP: Modulation of *in vitro* antitumor immunity in response to the biological activators of IL 1, γ-interferon, and GM-CSF in the peripheral blood of cancer patients receiving cytotoxic chemotherapy. Ann NY Acad Med 65:16-25, 1988.

45. Braun DP, Kessler H, Falk L, Paul D, Harris JE, Blaauw B, and Landay AL: Monocyte functional studies in asymptomatic HIV-infected individuals. J Clin Immunol 8:486-494, 1988.

46. Staren ED, Braun DP, Harris JE, Bines SD, and Economou SG: Lymphokine activated killer cell induction in the peripheral blood mononuclear cells of colon cancer patients. Surg Forum 39:447-449, 1988.

47. Ellis TM, Creekmore S, McMannis J, Braun DP, Harris JE, and Fisher RI: Appearance and phenotypic characterization of circulating Leu 19^+ cells in cancer patients receiving recombinant interleukin 2. Cancer Res 48:6597-6602, 1988.

48. Braun DP, Taylor ST IV, and Harris JE: Modulation of immunity in cancer patients by prostaglandin antagonists. Prog Clin Biol Res 288:444-448, 1989.

49. Creekmore SP, Harris JE, Ellis TM, Braun DP, Cohen II, Bhoopalam N, Jassak PF, Cahill MA, Canzoneri CL, and Fisher RI: A phase I clinical trial of recombinant interleukin 2 by periodic 24 hour infusions. J Clin Oncol 7:276-284, 1989.

50. Staren ED, Economou SG, Harris JE, and Braun DP: Lymphokine activated killer cell induction in tumor infiltrating leukocytes from colon cancer patients. Cancer 64:2238-2242, 1989.

51. Braun DP, Siziopikou KP, Casey LC, and Harris JE: The *in vitro* development of cytotoxicity in response to granulocyte/macrophage colony stimulating factor or γ-interferon in the peripheral blood monocytes of patients with solid tumors. Modulation

by arachidonic acid metabolic inhibitors. Cancer Immunol Immunother. 32:55-61, 1990.

52. Harris JE, and Braun DP: The effect of aspirin and other cyclooxygenase inhibitors on antitumor immunity. In: Aspirin towards 2000 (G. Fryers, editor). pp. 45-52. Royal Society of Medicine. 1990.

53. Siziopikou KP, Harris JE, Casey L, Nawans Y and Braun DP. Impaired Tumoricidal Function of Alveolar Macrophages from Patients with Non-small Cell Lung Cancer. Cancer, 68:1035-1044, 1991.

54. Braun, D.P., Gebel, H., Rana, N, Rotman, C, and Dmowski, W.P. Peritoneal Macrophage Function in Women with Endometriosis., Fertility and Sterility 57: 1203-1210, 1992.

55. Harris, J.E. and Braun, D.P.: Abnormal immunoregulation in human cancer at the actual tumor site. In "Combination Therapies. Biological Response Modifiers in the Treatment of Cancer and Infectious Diseases. A. Goldstein, ed. Plenum Publishing, New York, pp. 197-206, 1992.

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58. Braun, DP, Ahn, M, Chu, E, Casey, L.C., Harris, JE, Wilbanks, G, and Siziopikou, KP. The sensitivity of macrophages from Different Anatomical Sites of Cancer Patients to Arachidonic Acid Metabolism. Cancer Research 53: 3362-3368, 1993.

59. Braun, DP, Muriana M, Gebel H, Rotman C, Rana N and Dmowski WP. Monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. Fertility and Sterility 61: 78-85, 1994.

60. Braun, DP, Gebel H and Dmowski WP. Effect of danazol in vitro and in vivo on monocyte-mediated enhancement of endometrial cell proliferation in women with endometriosis. Fertility and Sterility 62: 89-95, 1994.

61. Gebel HM, Braun DP, Rotman C, Rana N and Dmowski WP. Decreased in vitro production of IgG in women with severe endometriosis. Amer. J. Reprod. Immunol. 29: 124-130, 1993.

62. Dmowski WP, Gebel HM and Braun DP. The role of cell-mediated immunity in pathogenesis of endometriosis. Acta Obstet Gynecol Scand 159: 7-14, 1994.

63. Gebel HM, Rana N, Braun DP, and Dmowski WP. Differential Expression of VLAB1 (CD29) on monocytes from patients with endometriosis. Amer J Reprod Immunol 34: 317-322, 1995.

64. Rana N, Braun DP, Rotman C, Gebel HM and Dmowski WP. Cytokine Synthesis by peritoneal macrophages in patients with endometriosis. Fertility Sterility,65:925-931,1996.

65. Braun DP, House R, Gebel HG, Rana N, and Dmowski WP. Cytokine synthesis by peripheral blood monocytes of patients with endometriosis. Fertility and Sterility 65: 1125-1129, 1996.

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108. Braun, DP, Dmowski, WP. Stimulation of eutopic and ectopic endoetrail cell proliferation by autologous peritoneal fluids with endometriosis is due to tumor necrosis factor-alpha (TNFα). Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontaior, Canada, September 25-30,1999.

109. Ding, J, Shen, J, Braun, DP, Rana, N, Fernandez, BB, Dmowski, WP. Apoptosis in Endometrial Glandular and Stromal Cells in Women with and without Endometriosis. Oral Presentation, ASRM/CFAS '99 Conjoint Annual Meeting, Ontario, Canada, September 25-30, 1999.

110. Shen, J., Ding, J, Braun, DP, Rana, N, Dowbrowski, A, Dmowski, WP. Endometrial Macrophages May Be Involved in the Regulation of Endometrial Apoptosis. Poster Presentation, ASRM/CFAS '99 Conjoint Annual meeting, Ontario, Canada, September 25-30, 1999.

111. Ding, J, Shen, M, Gogacz, M, Braun, DP, Rana, N., Dmowski, WP. Endometrial Apoptosis Is Inhibited In Vitro by TNFα in Women with Endometriosis and Is Stimulated by TNFα in Healthy Women. Journal of the Society for Gynecologic Investigation, 2000.

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113. Ding, J, Gogacz, Shen, M, Braun, DP, Rana, N, Dmowski. Expression of TNFα Receptor Type-I Gene in Endometrial Cells from Women with and without Endometriosis. Submitted to American Society For Reproductive Medicine, 56th Annual Meeting, October 21-25, 2000, San Diego, California.

UNIVERSITY INSTRUCTION:

Course Director

Principles of Immunology. University of Illinois, Dept. of Microbiology, 1977.

Biology of Cancer. College of Nursing, Rush Presbyterian St. Luke's Medical Center, 1982.

Tumor Immunology. Department of Immunology, College of Medicine, Rush Presbyterian St. Luke's Medical Center,1985.

The Basic Science of Oncology. Rush Cancer Institute. 1993-1995.

Lecturer

Basic and Clinical Immunology, Department of Immunology/Microbiology
Cellular Immunology, Department of Immunology/Microbiology

Tumor Immunology, Department of Nursing
Pathophysiology of Malignant Disease, Department of Pathology
Medical Oncology Didactic Course

Organizing

Medical Oncology Didactic Lecture Series
Medical Oncology Research Seminar Series
Oncology Fellow Basic Sciences Journal Club

Advisory

Graduate Students Matriculated (5)
Dissertation Advisory Committees (18)
As Chairman (2)
As Advisor (5)
As Member (11)
Other Department (2)
Other University (5; 4, University of Illinois, 1 Notre Dame)

UNIVERSITY COMMITTEES:

Student Judiciary Review
Academic Freedom, Chairman, 1994-1996
CED Review of Rush Medical College
Radiation Oncology Search Committee
General Surgery Search Committee
Department of Dermatology Search Committee
Department of OB/Gyn Search Committee
Faculty Council, 1996-1999.
Task Force on Interaction with Biomedical Industry, Chairman.
Academic Council

INVITED PRESENTATIONS:

1. Transfer of Plasmacytoma Immunity with Immune RNA Extracts from Tumor-bearing Balb/C mice. American Dental Association, Chicago, IL, 1977.

2. Principles of Cancer Immunology. University of Indiana School of Medicine. Gary, Indiana, 1978.

3. Synergy between Cytotoxic Chemotherapy and Anti-Tumor Immunity in Solid Tumor Cancer Patients. Department of Pathology, University of Illinois Medical Center, Chicago, IL, 1979.

4. Selective Effects of Cytotoxic Chemotherapy on Suppressor Cells in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1980.

5. Immunoregulatory Cell Function and Impaired Immunity in Patients with Gliomas. Department of Neurosurgery, Cook County Hospital, 1983.

6. Effect of Interferon Therapy on Prostaglandin-producing Suppressor Cells in Renal Cell Cancer Patients. Burroughs Wellcome, Research Triangle Park, North Carolina, 1984.

7. Changes in Prostaglandin Metabolism in Monocytes from Interferon-treated Cancer Patients.Department of Microbiology, University of South Florida, Tampa, Florida, 1985.

8. Potentiation of Immunity in Chemotherapy-Treated Cancer Patients. Northwestern School of Medicine, Cancer Center, Chicago, IL, 1985.

9. Prostaglandin-Producing Suppressor Cells in Cancer Patients and AIDS Patients. Department of Pathology, Loyola University School of Medicine, Chicago, IL. 1986.

10. Synergy between Chemotherapy and Immunity in Solid Tumor Cancer Patients. Roswell Park Memorial Institute, Grace Cancer Center, Buffalo, New York, 1986.

11. Modification of the Effects of Cancer Chemotherapy on Immune Responses in Lung Cancer Patients by Treatment with Piroxicam. Pfiser Pharmaceuticals, New Orleans, Louisiana, 1986.

12. Enhancement of Deficient Cellular Immunity in Head and Neck Cancer Patients Treated with Piroxicam. Pfizer Laboratories, Annaheim, California, 1987.

13. Modulation of Immunity in Cancer Patients by Prostaglandin Antagonists. Second International Conference on Immunity to Cancer. Williamsburg, Virginia, 1987.

14. Potential for Combining Cytotoxic Chemotherapy and Biological Response Modifiers in Cancer Patients. Illinois Cancer Council, Chicago, IL, 1988.

15. Monocyte Immunoregulatory Cell Function in HIV-Infected Patients. American Red Cross, Chicago, IL, 1988.

16. Lymphokine Activated Killer Cell Function in Tumor Infiltrating Leukocytes from Colon Cancer Patients. Illinois Cancer Council Symposium on Biological Response Modifiers, Chicago, IL, 1988.

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Exhibit B

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1515

Tumor inflammatory response induced by immunization with autologous melanoma cells conjugated to dinitrophenol(DNP).
D. Berd, M.J. Mastrangelo, C. Green, C. Clark, and E. Hart. Thomas Jefferson University, Philadelphia, PA 19107.

Treatment of melanoma patients with an autologous vaccine preceded by low dose cyclophosphamide (CY) induces delayed-type hypersensitivity (DTH) to melanoma cells, and in some cases, regression of metastatic tumors. Now, we are attempting to increase the efficiency of the process by immunizing with tumor cells conjugated to the hapten, DNP. Patients with metastatic melanoma were sensitized to DNP by topical application of dinitrochlorobenzene (DNCB). Two weeks later, they were injected with a vaccine consisting of 10-25x10(6) autologous, irradiated melanoma cells conjugated to DNP and mixed with BCG. CY 300 mg/M^2 IV was given 3 days before DNCB or vaccine. Of 4 patients evaluable so far, 3 have developed a striking inflammatory response in tumor masses after 2 vaccine treatments (8 weeks). Patient #1 developed erythema and swelling in the >50 large (1-3 cm) dermal metastases on her leg and lower abdomen, followed by ulceration and drainage of necrotic material, and some are beginning to regress. Biopsy showed infiltration with CD4+ and CD8+ T lymphocytes. Patient #2 developed erythema and swelling in the skin of her lower abdomen and groin overlying large (8 cm) nodal masses. These have not yet regressed, but have changed in consistency from rock-hard to fluctuant. Patient #3 exhibited moderate erythema in the skin overlying subcutaneous metastases. All 3 patients have developed DTH to both DNCB and to DNP-conjugated autologous lymphocytes. Although these results are preliminary, they suggest that this new strategy may represent a significant advance in the immunotherapy of human melanoma.

1516

Inhibition of Tumor-Induced Suppressor T Lymphocyte (Ts) Activity by Murine Interferon Beta (IFN-B).
Deepak M. Sahasrabudhe, University of Rochester Cancer Center, Rochester, NY, 14642

In some tumor models inhibition of Ts-activity is a prerequisite to successful immunotherapy. Based on our data in the DNFB model (J Exp Med 166:1573, 1987) the effect of IFN-B on P815 mastocytoma-induced Ts-activity was evaluated.

In this model, concomitant antitumor immunity (Tc) peaks by Day 10 and is down regulated by Ts by Day 15. Cytotoxicity generated after a mixed lymphocyte tumor culture (MLTC) correlates with in vivo immunity and suppression of cytotoxicity correlates with in vivo Ts-activity.

Tumors were initiated by injecting 2 x 10^6 P815 cells subcutaneously on Day 1. IFN-B (10U, 1000U, 5000U) or buffer were injected i.v. every other day x 5 dose. starting on Day 5. On Day 16, MLTC's were set up. Five days later a cytotoxicity assay was performed against ^{51}Cr labelled P815 cells. % specific lysis is shown. Numbers in parenthesis represent the dose of IFN-B.

E:T	Tc + Naive		Tc Ts	Tc +Ts	Ts (10)	Tc +Ts (10)	Ts (1000)	Tc +Ts (1000)	Ts (5000)	Tc +Ts (5000)
50:1	88	81	0	19	6	22	23	20	81	84
25:1	84	76	0	12	2	21	1	21	63	75
12:1	78	79	2	15	3	24	6	23	58	81
6:1	70	69	1	7	0	9	0	20	38	64
3:1	56	55	0	8	1	13	0	12	21	48

Treatment with IFN-B 5000U every other day x 6 doses abrogated Ts-activity without adversely affecting cytotoxicity. IFN-B may be a useful adjunct in the immunotherapy of selected tumors.

1517

Anti-idiotype monoclonal antibody immunization therapy of cutaneous T cell lymphoma.
Chatterjee, M., Foon, K., Seon, B.K., Barcos, M. and Kohler, H., Roswell Park Mem. Inst., Buffalo, NY 14263, and UCSD, San Diego, CA 92161.

Cutaneous T cell lymphoma (CTCL) is an indolent non-Hodgkin's lymphoma which is not cured by standard therapies once it reaches advanced stage. A novel approach to therapy is to use internal image anti-idiotype (Id) mAb as antigen (Ag) substitute for the induction of immunity. We have generated anti-Id mAb (Ab2) binding to a hybridoma SN2 (Ab1), which recognizes a unique glycoprotein, gp37, expressed by a subset of human leukemic T cells (J. Immunol. 139:1354, 1987). At least 2 of these Ab2 may indeed carry the internal image of the gp37 Ag (J. Immunol. 141:1398, 1988). Recently, we investigated the distribution of gp37 Ag by a sensitive immunoperoxidase staining method using mAb SN2. SN2 had a high specificity for T-leukemia/lymphoma cells and did not react with any normal adult tissues tested including thymus, lymphocytes, bone marrow cells, spleen, liver, kidney, lung, brain, heart, etc. CTCL cells from 5/6 out of 6 patients were strongly positive for gp37 Ag with intense surface membrane staining. The binding of radiolabeled SN2 to CTCL cells was studied for inhibition in the presence of the anti-Id mAbs 4EA2 and 4DC6 which mimic the gp37 Ag. Both clones inhibited the binding 100% and 80% respectively at a concentration of 50 ng. We also generated a murine Ab3 mAb (anti-anti-Id) by immunizing mice with the anti-Id mAb (Ab2). This Ab3 mAb reacts with CTCL cells in an identical fashion as the original Ab1 (SN2). Collectively, these data suggest that Ab2 4EA2 and 4DC6 may be useful for active immunotherapy of CTCL patients. We also plan to study the CTCL patients in a phase I clinical trial to determine the effects of this type of therapy on various components of the immune system (both humoral and cellular) and try to identify the criteria to select patients who may benefit from anti-idiotype vaccine therapy.

1518

Syngeneic murine monoclonal antiidiotypes bearing the internal image of a human breast cancer associated antigen.
J. Schmitz and H. Ozer. The Dept. of Microbiology, S.U.N.Y. at Buffalo, Buffalo, NY 14214 and the Division of Medical Oncology, The Univ. of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

According to Jerne's network theory, some antiidiotypes (Ab2) mimic external antigens recognized by specific antibodies (Ab1) and may be used in place of antigen for immunization. The murine monoclonal antibody F36/22 (IgG3, κ), specific for ductal carcinoma antigen (DCA) was used to generate syngeneic monoclonal antiidiotypes bearing the internal image of DCA. Female BALB/c mice were inoculated intraperitoneally every other week with 100 μg of F36/22 coupled to keyhole limpet hemocyanin; the first time in complete Freund's adjuvant and subsequently in incomplete adjuvant. Splenic lymphocytes were fused with the murine cell line P3X63 Ag8.653 3 days after the fourth immunization using 50% polyethylene glycol (m.w. 3000). Two hybrids, MTO-1 and MTO-2, were selected based on the ability of culture supernatants to bind to F36/22 but not to the control antibody 2A31F6 (IgG3, κ) in an enzyme linked immunosorbent assay (ELISA) and cloned by limiting dilution. Paratope specificity of Ab2 was demonstrated in two ELISA assays. First, the binding of labeled F36/22 to DCA was inhibited 100% and 75% by 1.6 μg of MTO-2 and MTO-1 respectively. Second, the binding of labeled Ab2 to Ab1 was inhibited by purified DCA. MTO-1 neither enhances nor inhibits the binding of labeled MTO-2 to Ab1 although in the presence of MTO-2, binding of labeled MTO-1 is enhanced by 100% indicating that these Ab2 recognize distinct idiotopes. Rabbits immunized biweekly with MTO-1 or MTO-2 developed antibodies that bound specifically to DCA demonstrating that MTO-1 and MTO-2 bear the internal image of DCA. These data suggest that MTO-1 and MTO-2 could potentially be utilized to immunize high risk patients against progression or development of DCA positive tumors.

Exhibit C

TUMOR VACCINATION

Jules E. Harris, MD
Donald P. Braun, PhD
Rush Medical College of Rush University

An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.

Tumor vaccination is an active, specific immunotherapy for malignant disease. It may be defined as "the administration of tumor cells, modified tumor cells, or tumor-cell surface-membrane preparations to stimulate or to augment various components of antitumor immunity to induce tumor regression or to prolong tumor remission achieved by conventional therapy."[1]

Vaccines also may be considered a type of biologic response-modifier therapy. The approach is based on the belief that the host is capable of mounting an effective immune response against tumors if appropriately stimulated, a belief that was first advanced around the turn of the century.[2] The concept of immunologic surveillance, which evolved some 40 to 50 years later, suggested that the human host was capable, under certain circumstances, of rejecting a tumor essentially in the same manner as a homograft was rejected.[3]

The first attempt to vaccinate humans against cancer was undertaken in 1902.[4] In this initial attempt, fluid was extracted from tumors in patients with advanced disease.[5] Over the next 50 years, a great variety of tumor cell preparations obtained from autologous or allogeneic tumors were used, generally to treat patients with advanced disease.

Fresh interest in the clinical potential of tumor vaccination was stimulated in the 1950s and 1960s by experimental studies conducted in syngeneic rodents. These demonstrated unequivocally that chemically induced and virally induced tumors had both shared and uniquely individual tumor-specific transplantation antigens (TSTAs).

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens (TAAs) rather than against TSTA. Also, TAAs were found on embryonic cells and tumor cells. The "unique antigens" on human tumor cells appear to result from tumor cell dedifferentiation for display of a partial embryonic-cell-membrane antigenic profile. Other TAAs arise as a consequence of the modification of normal "self" antigens producing an "altered self" phenotype.

TABLE 1

Antitumor Immune Mechanisms

- Activated macrophage cytotoxicity
- Cytotoxic T cells
- Natural killer (NK) cells
- Lymphokine-activated killer cells
- Humoral antibodies (complement dependent)
- Antibody-dependent cellular cytotoxicity (complement-independent, macrophage, neutrophil or NK-cell-dependent)

MANY UNRESOLVED CLINICAL ISSUES

The principal human immune responses to tumor antigens are listed in Table 1. It has not been definitively established which of these immune responses, alone or in combination, are the most important in a host response to cancer, nor is it clear which should be targeted for stimulation with tumor vaccination. There are many unresolved issues pertaining to the actual vaccine formulation that need to be addressed; some of these depend on whether the vaccine formulation is based on intact tumor cells (Table 2) or tumor cell extracts or products (Table 3).

In addition to vaccine formulation, several other questions remain:

- Should cellular extracts or whole cells be mixed with immunomodulating adjuvants

to increase tumor vaccine immunogenicity?

- Should tumor vaccines with or without adjuvants be used alone or in combination with cytotoxic drugs that can modulate or suppress undesirable immune responses?
- Should cytokines be used to augment immune responses to a vaccine?
- Is there a place for tumor vaccination in patients with advanced cancer?
- What is the appropriate dose, schedule, and route of administration for effective tumor vaccination?
- What measurement or surrogate biologic end point can be used to assess the biologic effectiveness of the vaccine?
- Will the immune response against TAA produced by a human tumor vaccine be selective and specific for tumor cells or will autoimmune reactions against normal cells be a possible toxicity associated with vaccination?

Advances in molecular genetics and the availability of monoclonal antibody reagents now make it possible to purify cells and cell components with defined and unique antigenic characteristics for use in human tumor vaccines. However, a number of the promising clinical trials of tumor vaccination conducted in the 1970s and 1980s used relatively simple and empiric methods of tumor vaccination preparation.

In one study, surgically resected stage I and II lung cancer patients were treated with a vaccine prepared from allogeneic tumor cells.[6] Cell membranes from viable tumor cells were subjected to low-frequency sonication and the soluble material separated with Sephadex G-200. Polyacrylamide gel electrophoresis was used to purify protein band material, which could elicit delayed hypersensitivity reactions (DHRs) in lung cancer patients. This material was administered intracutaneously in combination with Freund's complete adjuvant (FCA) in a series of three injections at monthly intervals beginning about 1 month after surgery.

Pilot studies suggested that this form of therapy delayed or prevented tumor recurrence. The approach was tested in a large multicenter clinical trial, which found no difference in survival between control patients and patients treated with FCA alone or with FCA and tumor antigen.[6] No autoimmune toxicity was noted during the course of these studies. Peripheral blood monocytes producing excessive amounts of prostaglandins appeared in the circulation prior to clinical relapse in patients who failed in all three arms of the study.

Further analysis of this clinical trial, however, suggests that a survival benefit may have been obtained in the fraction of patients in whom careful attention was paid to thorough homogenization of tumor antigen in the FCA. Vaccinated long-term survivors also may have developed more intense DHRs to tumor antigen. The methods employed in this study, although important and innovative, need to overcome the problematic nature of the technique's purification process and reproducibility (Table 3) before wider application in humans is feasible.

A tumor vaccination study in patients with surgically resected Dukes B_2 through C_2 colorectal cancer was conducted, based on rigorously evaluated preclinical experimental animal data in which requirements for effective immunotherapy were established.[7] An elegant series of studies of a guinea pig line-10 hepatocarcinoma model showed convincingly that bacillus Calmette-Guérin (BCG) admixed with syngeneic tumor cells could induce sufficient systemic immunity to elimi-

TABLE 2

Critical Issues for Whole-Tumor-Cell Vaccines

Should autologous or allogeneic cells be employed?
Should cells be obtained from fresh surgical specimens or from tissue cell lines?
Should cells first be irradiated to maintain their membrane integrity but prevent their proliferation?
How can the reproducibility of vaccine preparation be assured?
How can whole tumor cells be used that are gene modified for the following phenotypic changes (individual or in combination) to enhance immunogenicity: (1) expression of HLA class I or II antigens and/or adhesion molecules; (2) secretion of immunomodulatory stimulating cytokines, such as interleukin-2 or tumor necrosis factor; and (3) secretion of chemotactic cytokines?

The concept of immunologic surveillance... suggested that the human host was capable under certain circumstances of rejecting a tumor essentially in the same manner as a homograft was rejected.

Humoral and cellular immune responses were shown to exist in patients with cancer; these were found to be directed against tumor-associated antigens....

nate a limited metastatic disease burden.[8] These studies controlled for variables such as the number and viability of tumor cells, and ratio of viable BCG organisms to tumor cells. In the pilots that evolved from these trials, patients were randomized to a control arm or were vaccinated with their own tumor cells, obtained from surgical specimens at the time of operation and cryopreserved until thawed and irradiated prior to use.[9] Treated patients underwent a schedule of three intradermal vaccine treatments weekly beginning 4 to 5 weeks after tumor resection.

The first two vaccine preparations consisted of irradiated cells and BCG; the third vaccine preparation was composed of irradiated tumor cells alone. Vaccinated patients developed augmented DHR to their autologous tumor cells with greater frequency than nonvaccinated patients. A DHR increase to autologous normal intestinal mucosa cells was not seen. An Eastern Cooperative Oncology Group trial is now evaluating this approach for surgically resected Dukes B_2 and B_3 patients.

FIRST TRIALS WITH HUMAN TUMOR VACCINES

These studies, the first truly large, randomized, controlled, multi-institutional clinical trials of human tumor vaccines for solid tumors, are examples of the use of whole cells and cell extracts for human tumor-vaccine preparation. In each case, an adjuvant substance was added to enhance the immunogenicity of the vaccine.

An autologous whole-cell vaccine has been shown to induce DHR to the whole-cell component, as well as significant regression of metastasis in patients with metastatic malignant melanoma.[10] Patients received cyclophosphamide before the vaccine in an attempt to modulate the activity of suppressor T-lymphocytes. The vaccine was prepared by methods similar to those previously described[7] and combined with BCG.

Other investigators have also used cyclophosphamide to inhibit suppressor T-lymphocyte activity prior to the administration of a malignant melanoma vaccine.[11] The vaccine preparation consisted of mechanically disrupted allogeneic tumor cells from melanoma cell lines. The concentration of vaccine was standardized in the preparation through measurements of a melanoma-associated antigen.

Measurement was performed by binding inhibition enzyme immunoassays using a monoclonal antibody. The vaccine was given subcutaneously with an adjuvant consisting of detoxified endotoxin (monophosphoryl lipid A) mycobacterial cell wall skeleton and squaPane oil. In this trial, regressions of disease were seen in patients with metastatic disease.

TABLE 3

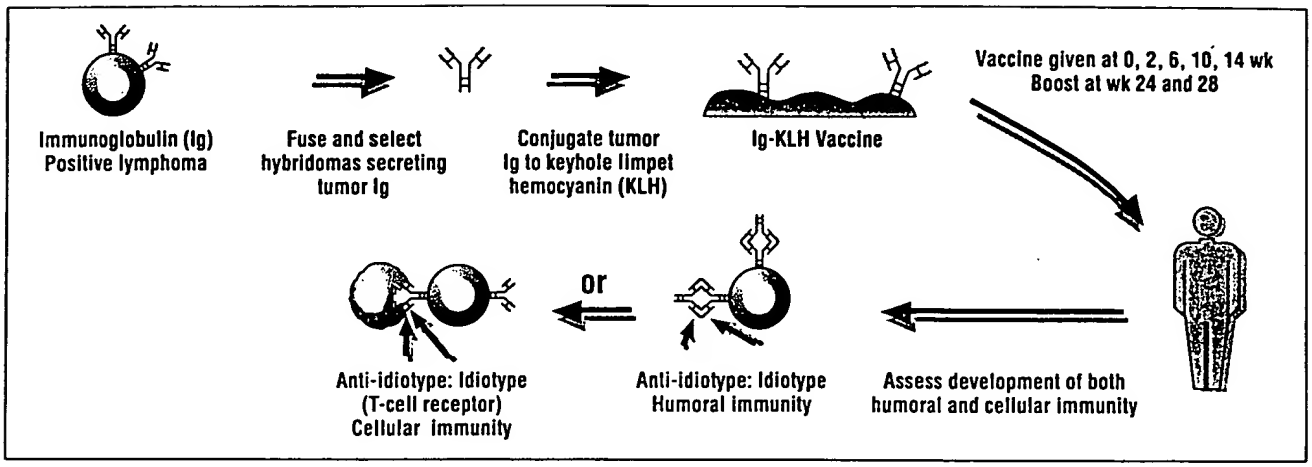
Critical Issues for Tumor-Cell-Extract Vaccines

What method of antigen extraction should be employed?
Should material be derived from a single source or should pooled material from a number of sources be used?
What methods are to be used to identify material in cellular extracts that will produce the most effective stimulation of antitumor immune responses?
Should extracted material be separated from HLA antigens that are present on both normal and malignant cells?
How can reproducibility of vaccine preparation be assured?

VIRAL ONCOLYSATES FOR HUMAN TUMOR VACCINATION

The use of viral oncolysates for human tumor vaccination combines the potential immunogenic benefit of whole cells with the value of cell extracts.[12] Viral oncolysates are homogenates of virus-infected cells. The virus in the mixture is believed to have an adjuvant rather than an antigenic role. Allogeneic and autologous viral oncolysates have been used in human immunotherapy. Influenza virus and vaccinia virus have been most frequently used in the preparation of viral oncolysates since the first report of this procedure in 1974.[13]

Pilot studies have suggested a protective or therapeutic benefit for viral oncolysates in gynecologic cancer, melanoma, and sarcoma. However, these reports must be considered anecdotal until larger randomized investigations are conducted.



It may be possible to vaccinate against some human cancers by immunizing against those few viruses presently known to be associated with cancer in humans. Hepatitis B virus infection is associated with the development of primary hepatocellular cancer. Immunizing against this virus will prevent its hepatic damage and may reduce the incidence of associated cancer.[14]

Finally, one of the most innovative approaches to tumor vaccination that has been developed relies on the use of idiotypic molecules that reiterate the molecular configuration of tumor-associated antigens. This approach is based on principles that predict that the variable regions of immunoglobulins and T-cell receptors that are responsible for antigen recognition are themselves capable of provoking both B-cell and T-cell immunity.[15]

These concepts led other investigators to vaccinate patients who have B-cell lymphoma with the autologous immunoglobulins from each patient's tumor following cytotoxic chemotherapy[16] (Figure). Vaccinated patients developed either humoral immunity, cellular immunity, or both; in the two patients with measurable disease, complete tumor regression was observed. These preliminary results demonstrate the feasibility of idiotypic vaccination for B-cell and T-cell malignant diseases and suggest that similar approaches might also be developed for nonlymphoreticular malignancies.

FIGURE

Strategy for idiotype vaccination. Source: Adapted from Kwak LW, Campbell MJ, Czerwinski DK, Hart S, Miller R, Levy R. Induction of immune responses in patients with B cell lymphoma against surface immunoglobulin idiotype expressed by their tumors. *N Engl J Med.* 1992; 327:1209-1215.

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Exhibit D

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[CANCER RESEARCH 38, 204-209, January ...8]

Immunotherapy of Established Micrometastases with *Bacillus Calmette-Guérin* Tumor Cell Vaccine[1]

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ABSTRACT

We evaluated the use of *Bacillus Calmette-Guérin* admixed with tumor cells as a vaccine to induce systemic tumor immunity for therapy of subclinical (micrometastatic) disease. In several experiments inbred strain 2 guinea pigs were given i.v. injections of either 10^4, 10^5, or 10^6 syngeneic L10 hepatocarcinoma cells, and initial vaccinations were administered either 1 or 4 days after tumor inoculation. Variables in vaccine preparation, such as ratio of viable *Bacillus Calmette-Guérin* organisms to tumor cells, procedures for freezing the tumor cells, X-ray treatment of tumor cells, and vaccination regimen were evaluated. The studies demonstrated that under defined conditions nontumorigenic vaccines of *Bacillus Calmette-Guérin* and tumor cells can cure the majority of animals of otherwise lethal visceral micrometastases.

INTRODUCTION

The strategy of immunotherapy for cancer in experimental animal models and humans is limited by many factors including the stage, type, and location of the tumor; the level of antigenicity of the tumor cells; and the status of the host immune response. Clinical immunotherapy has been proceeding with relatively limited guidance from experimental animal models. Of the several approaches to immunotherapy of localized tumor and/or disseminated minimal residual tumor, immune potentiation by microbial agents has received the greatest attention. The most encouraging experimental and clinical data to date have resulted from protocols consisting of bacterial vaccines or nonspecific immunostimulants, primarily *Mycobacterium bovis* strain BCG,[2] administered i.t. (17, 18, 21) or systemically either alone (7, 8, 16) or admixed with tumor cells in the form of a vaccine (22, 23). One impetus for the use of BCG in immunotherapy has been the development of an experimental system that meets some of the requirements of a model to study an established tumor with regional lymph node metastasis (19). It has been demonstrated that regression of transplanted syngeneic hepatocarcinomas growing in the skin of inbred strain 2 guinea pigs and elimination of regional lymph node metastases are achieved in the majority of animals after i.t. injection of viable BCG (12, 26). This particular aspect of immunotherapy in the guinea pig model, although intriguing, is very limited with respect to the type, stage, and location of the tumor as well as with respect to the route of administration of BCG. Nevertheless, the initial studies established 1 fact that has broad implications. During BCG-mediated tumor regression and elimination of regional lymph node metastases, there is the development of systemic cell-mediated tumor immunity demonstrated by rapid rejection of a second tumor challenge several weeks after BCG treatment (11, 25, 27). This is a very important aspect of the model since it is known that, at the tumor stage when BCG administration is optimally effective, surgical excision of the tumor and regional lymph node would also be curative. However, no significant development of tumor immunity is achieved with surgery alone.

We recently demonstrated the effectiveness of tumor immunity induced by i.t. injections to eliminate artificially produced distant tumor foci (9, 10). This aspect of the BCG therapy model becomes important when one considers that adjuvant immunotherapy has been primarily tested in cancers for which control of primary tumors is available with surgery, radiotherapy, and/or chemotherapy, but where there is a substantial rate of relapse. Recurrence is usually thought to be due to a small number of residual tumor cells. Adjuvant immunotherapy is intended to eradicate the residual tumor cells by enhancing immunological mechanisms. However, based on all that we have learned, the translation to humans of the results of i.t. BCG injection in the guinea pig model would require careful attention to certain aspects of the treatment. These include tumor stage, dose, injection, route, regimen, and source of BCG. This is not always possible in human cancer, for which immunotherapy is often used for advanced cancer after other forms of treatment have failed. In addition, this model is inappropriate for cases in which i.t. injections are not possible.

An important advance in this guinea pig immunotherapy model would be to achieve effective systemic tumor immunity without the i.t. injection of BCG. We have approached this problem by systematically evaluating the ability of vaccines of BCG admixed with tumor cells to eliminate a disseminated tumor burden. Although previous attempts at BCG-tumor cell vaccine immunotherapy both in inbred guinea pigs (3) and in humans (see Ref. 20 for review) have been limited and somewhat discouraging, relatively little has been done to determine the optimal conditions for vaccination. Here we investigate a number of variables such as the ratio of viable BCG organisms to tumor cells, the freezing procedures, the X-ray treatment of cells, and the vaccination regimen. Although these factors cannot possibly be investigated systematically in humans for ethical reasons, they can be studied in the guinea pig model. Our studies demonstrate that, under defined conditions,

[1] Research sponsored by the National Cancer Institute under Contract NO1-CO-25423 with Litton Bionetics, Inc.

[2] The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; i.t., intratumoral; i.l., intralesional; L10, line 10 hepatocarcinoma cells; HBSS, Hanks' balanced salt solution; i.d., intradermal; PPD, purified protein derivative of *Bacillus Calmette-Guérin*.

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nontumorigenic vaccines of BCG and tumor cells can cure the majority of animals with lethal disseminated tumors established as visceral micrometastases.

MATERIALS AND METHODS

Animals. Inbred male Sewall Wright strain 2 guinea pigs were obtained from the Frederick Cancer Research Center Animal Breeding Section. These guinea pigs were shown to be histocompatible by skin grafting. They were housed 6 to 10 per cage and fed Wayne guinea pig chow and kale; they weighed 400 to 500 g at the beginning of the experiments.

Tumors. Induction of primary hepatocarcinomas in strain 2 guinea pigs after they were fed the water-soluble carcinogen, diethylnitrosamine, was described previously (19). The antigenic and biological properties of the transplantable ascites tumors developed from the primary hepatocarcinomas have also been described (28).

Ascites hepatocarcinoma cells, L10, were harvested and washed 3 times in HBSS and diluted to desired concentrations. One-ml doses of L10, ranging from 10^4 to 10^6 cells/dose, were injected into the dorsal vein of the penis, producing artificial vascular metastasis. Injections of 10^4 cells resulted in the death of approximately 70 to 80% of the animals, whereas 10^5 and 10^6 cells were fatal to all animals. The times of death varied as a function of dose, and all animals died as a result of metastasis to the lung, mediastinal lymph nodes, and hilar lymph nodes with concurrent visceral metastases.

BCG. *M. bovis* strain BCG (Phipps strain TNC 1029) was obtained from the Trudeau Institute (Saranac Lake, N. Y.). Preparations of BCG, stored at −70°, were rapidly thawed in a 37° water bath and diluted to proper concentrations.

Vaccine Preparation. The L10 tumor was maintained by i.p. passage in guinea pigs. Ascites cell preparations were removed and washed in HBSS. The L10 cells used in vaccine preparation were either fresh or frozen and thawed.

In preparation for freezing, the cells were concentrated and suspended in an equal volume of chilled 15% dimethyl sulfoxide plus 10% fetal calf serum-HBSS solution. The final suspension was 2 to 6 × 10^7 cells/ml. Two-ml aliquots of the L10 cell suspension were frozen at controlled rates in a Linde BF4 Biological Freezer at −1°/min to the critical freezing point, flash-frozen through the heat of fusion, and continued at −1°/min to a final temperature of −60°. The rate of freezing was monitored on a Honeywell Electronic III. The vials were stored in liquid nitrogen. The rationale for this method of freezing has been described in detail elsewhere (14, 15). The vials were rapidly thawed in a 37° water bath. Frozen-thawed cells were slowly diluted to 50 ml in HBSS, washed once, and resuspended in preparation for X-irradiation. Suspensions of fresh and frozen-thawed cells were X-irradiated in 50-ml beakers on ice. X-irradiation was performed with a Phillips MG 301 X-irradiation unit at 500 R/min. A total X-irradiation dose of 20,000 R was achieved. Cell viability counts were performed with the use of the trypan blue dye exclusion test, and viability after irradiation of either fresh or frozen-thawed cells was generally 90%, with less than 10% variation between the fresh or frozen-thawed cells.

BCG (10^9 organisms/ml) was added in equal volume to viable L10 (10^8 cells/ml) for a vaccine ratio of 10:1. A vaccination consisted of an i.d. injection of 0.2 ml. For ratios of 1:10, BCG (10^9 organisms/ml) was diluted 1:100 in HBSS, and aliquots were mixed with 10^8 viable L10 cells/ml. These vaccinations also consisted of an i.d. injection of 0.2 ml. All vaccinations were performed less that 1 hr after the BCG-tumor cell mixtures were prepared.

In preliminary vaccination experiments, the L10 cells were irradiated with 12,000 R; however, we noticed that, although this irradiated cell preparation was not tumorigenic when admixed with BCG, it was tumorigenic when administered i.d. in the absence of BCG. We were concerned that any growth of 12,000-R X-irradiated L10 cells in the skin might preempt developing tumor immunity and thus render the treatment ineffective against disseminated tumor. Therefore, 20,000-R X-irradiation was used in all subsequent experiments with L10 cells in BCG-tumor cell vaccines. Animals were given i.v. injections, in the dorsal vein of the penis, of either 10^4, 10^5, or 10^6 L10 cells in 1-ml volumes. All vaccinations were given i.d., beginning in the upper right dorsal quadrant. Successive vaccinations were given in different sites or i.l. in the previous vaccination site. Vaccinations were performed either 1 and 7 days or 4 and 10 days after i.v. L10 injection.

RESULTS

An i.v. dose of 10^4 L10 tumor cells does not lead to the death of all guinea pigs. Approximately 25% of the animals will survive clean injections where leakage did not occur to the regional site. This inoculum is the optimal dose for assessing the influence of the nonspecific side effects of vaccination on tumor cell arrest, the extravasation and establishment in organs, and the immunologically specific effects of the vaccine. Thus, at this initial tumor cell dose of 10^4, vaccinations were performed at either 1 and 7 or 4 and 10 days after i.v. injections of L10.

Several modes of vaccination as well as 2 ratios of viable BCG to tumor cells were tested in guinea pigs given i.v. injections of 10^4 L10 cells. The BCG-tumor cell ratios were 10^8 BCG or 10^6 BCG admixed with 10^7 L10. These were administered as either a single vaccination, a single injection of BCG-L10 vaccine followed 6 days later by an i.l. injection of L10 into the previous vaccination site, a single injection of BCG-L10 vaccine followed 6 days later by an injection of L10 alone on the opposite side, or 2 separate injections of BCG-L10 vaccine. Also, the efficacy of frozen L10 cells was compared to that of fresh L10 cells. The results are shown in Table 1.

Compared to the untreated tumor-bearing guinea pigs, no significant difference in survival was detected in animals treated with 2 i.d. injections of BCG or tumor cells alone, regardless of whether the initial treatment was performed 1 or 4 days after i.v. injection of L10.

Single BCG + L10 vaccinations at ratios of 1:10 or 10:1 did not confer significantly greater protection than did vaccinations of BCG alone, tumor cells alone, or nontreated controls. Furthermore, these 2 BCG:L10 ratios could not be associated with significant differences in survival of animals given i.v. injections of 10^4 tumor cells, regardless

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Table 1

Survival of guinea pigs given i.v. injections of 10^4 syngeneic L10 hepatocarcinoma cells

This experiment was terminated at 280 days after tumor injection; all nontreated controls died by 120 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test

Treatment[a]	No. of survivors/total no. of animals/group at following vaccination times after i.v. injection of tumor	
	Days 1 and 7	Days 4 and 10
None	3/12	
(10^8 BCG) (10^8 BCG)	3/12	3/10
(10^7 L10) (10^7 L10)	2/10	
(10^8 BCG + 10^7 L10)[b]	4/10	4/10
(10^8 BCG + 10^7 L10)[b]	2/10	
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	8/10	8/10
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	9/10	9/10
(10^8 BCG + 10^7 FL10[c]) (10^7 FL10 i.l.)	8/10	
(10^8 BCG + 10^7 L10) (10^7 L10)	10/10	10/10
(10^8 BCG + 10^7 L10) (10^7 L10)	10/10	9/10
(10^8 BCG + 10^7 L10) (10^8 BCG + 10^7 L10)	10/10	10/10
(10^8 BCG + 10^7 L10) (10^8 BCG + 10^7 L10)	9/10	10/10

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

of the vaccination schedule. Compared to those animals that received single vaccinations of BCG + L10, BCG, or tumor cells alone and compared to the nontreated controls, significant differences in survival were achieved in tumor-bearing guinea pigs that received the second vaccination of either L10 i.l. ($p < 0.03$), L10 on the opposite side ($p < 0.01$), or BCG-L10 mixture ($p < 0.01$). From 60 to 100% of the animals survived in these treatment groups, regardless of whether the initial vaccine was administered 1 or 4 days after i.v. L10 injection. No significant differences in efficacy were detected between fresh L10 cells and frozen L10 cells.

At 280 days, representative groups of the survivors either were tested for tumor immunity by measurement of rejection of i.d. challenge of 10^6 L10 cells or were killed and autopsied for gross and histological examination for residual tumor. None of the animals autopsied had any evidence of residual tumor. Tumor challenge groups varied in their ability to reject contralateral challenge as a function of treatment. All nontreated controls or groups that had been treated with BCG or tumor cells alone failed to reject contralateral challenge, indicating that these animals were not tumor immune at 280 days after treatment. Seventy to 90% of the survivors in the various multiple vaccination groups rejected contralateral challenge; however, no significant difference in tumor immunity, as measured by contralateral challenge, could be detected among these treatment groups. These data demonstrate that animals that survived after treatment with ineffective modes of vaccination were not tumor immune, whereas significant protection as well as long-term tumor immunity was conferred on those animals that received efficacious modes of vaccination.

Injections of 10^5 or 10^6 syngeneic L10 cells i.v. are routinely fatal in strain 2 guinea pigs. Vaccinations of BCG alone or tumor cell alone conferred no protection in these tumor-bearing guinea pigs when the animals were given vaccinations 1 and 7 days after i.v. tumor inoculation (Table 2). Survival in all treatment groups was a function of the BCG:L10 cell ratio. Without exception, in guinea pigs given 10^5 or 10^6 cells i.v., a vaccine containing BCG:L10 cells in a ratio of 10:1 yielded significant protection, whereas a ratio of 1:10 was ineffective. Thus, the ratio of viable BCG organisms to tumor cells is a critical factor in the efficacy of the vaccine, and a large amount of BCG is beneficial in the initial vaccination. No significant difference in protection could be detected when the group that received a single BCG + L10 vaccination (10:1) was compared to a similar treatment group that received a second i.l. L10 injection. In contrast, survival was achieved in those animals that received a second injection of L10 alone or BCG + L10 on the opposite side ($p < 0.02$ or $p < 0.01$, respectively). In 2 groups of animals given i.v. injections of 10^6 L10, no significant difference in protection was detected when frozen-thawed L10 was used in the vaccine in place of the fresh L10.

One important consideration was whether BCG-immune guinea pigs could generate effective tumor immunity after

Table 2

Survival of guinea pigs given i.v. injections of 10^5 or 10^6 syngeneic L10 hepatocarcinoma cells

These experiments were terminated at 240 days after tumor injection. All nontreated controls in the 10^5 group died by 95 days, and all nontreated controls in the 10^6 group died by 77 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	No. of survivors/total no. of animals/group at following i.v. tumor cell dose	
	10^5	10^6
None	0/10	0/10
(10^8 BCG) (10^8 BCG)	0/10	0/10
(10^7 L10) (10^7 L10)	0/10	0/10
(10^8 BCG + 10^7 L10)[b]	1/10	0/10
(10^8 BCG + 10^7 L10)[b]	2/10	0/10
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	1/10	
(10^8 BCG + 10^7 L10) (10^7 L10 i.l.)	5/10	
(10^8 BCG + 10^7 FL10[c]) (10^7 L10 FL10 i.l.)	5/10	
(10^8 BCG + 10^7 L10) (10^7 L10)	1/10	0/10
(10^8 BCG + 10^7 L10) (10^7 L10)	8/10	3/10
(10^8 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10	1/10
(10^8 BCG + 10^7 L10) (10^8 BCG + 10^7 L10)	9/10	8/10
(10^8 BCG + 10^7 FL10) (10^8 BCG + 10^7 FL10)	9/10	

[a] Treatments were administered 6 days apart on opposite sides as described in "Materials and Methods."
[b] Vaccinations were administered as single sequential injections.
[c] FL10, frozen-thawed L10.

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BCG + L10 vaccination. Normal guinea pigs as well as guinea pigs previously immunized to BCG and shown to be PPD positive by skin testing were given i.v. injections of 10^5 L10 cells. In this particular experiment vaccinations were performed 4 and 10 days after i.v. tumor inoculation. Two modes of vaccination, BCG + L10 (10:1) at Days 4 and 10 and BCG + L10 (10:1) at Day 4 followed by i.l. L10 at Day 10, were compared in PPD-positive and PPD-negative guinea pigs. Regardless of whether or not the animals were PPD positive, the 2 modes of vaccination conferred significant protection ($p < 0.01$) and did not differ significantly (Table 3).

We next investigated whether multiple BCG + L10 vaccinations at either the 10:1 or 1:10 ratios would improve survival in comparison to single or sequential vaccinations. Guinea pigs were given i.v. injections of 10^6 L10 cells and vaccinated on either 1 day, 1 and 7 days, or 1, 7, and 14 days after i.v. tumor inoculation. Treatments consisted of a single vaccination of BCG + L10 followed by L10 alone or by BCG + L10, 2 simultaneous BCG + L10 vaccinations followed by 2 simultaneous injections of L10 alone or of BCG + L10, or 3 sequential vaccinations of BCG + L10. The results are shown in Table 4.

Initial BCG + L10 vaccinations at a ratio of 1:10 were ineffective regardless of the vaccination schedule. Significant protection ($p < 0.01$) was achieved with all initial vaccinations at ratios of 10:1, but no significant increase in survival was achieved by multiple or sequential vaccinations.

Table 3

Survival of guinea pigs given i.v. injections of 10^5 syngeneic L10 hepatocarcinoma cells: effectiveness of vaccination in BCG-immune guinea pigs

Guinea pigs were given i.d. injections of 10^6 BCG and skin tested with PPD 21 days after immunization; 2 weeks later animals were given i.v. injections of 10^5 L10. The experiment was terminated 270 days after tumor injection, and all nontreated controls died by 128 days after tumor injection. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

PPD sensitivity	Treatment at 4 and 10 days	Survivors/total no. of animals/group
−	None	0
+	None	0
−	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
+	(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	6/10
−	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	2/10
+	(10^6 BCG + 10^7 L10) (10^7 L10 i.l.)	6/10

Table 4

Survival of guinea pigs given i.v. injections of 10^6 syngeneic L10 hepatocarcinoma cells: effect of multiple vaccinations

This experiment was evaluated at 120 days after i.v. tumor injection, and all animals in the nontreated or single vaccination control groups died by 70 days. Significance of differences in survival was calculated by the Fisher 2-tailed exact test.

Treatment[a]	Survivors/total no. of animals/group
None	0/13
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10)[b]	0/10
(10^6 BCG + 10^7 L10) (10^7 L10)	0/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)[c]	1/9
(10^6 BCG + 10^7 L10) (10^7 L10)	3/10
2(10^6 BCG + 10^7 L10) 2(10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	1/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	1/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	5/10
2(10^6 BCG + 10^7 L10) 2(10^6 BCG + 10^7 L10)	6/10
(10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10) (10^6 BCG + 10^7 L10)	4/9

[a] Treatments were administered 6 days apart on opposite sides, as described in "Materials and Methods."
[b] Vaccinations were administered as single injections.
[c] Two simultaneous injections.

DISCUSSION

Vaccines consisting of tumor cells admixed with BCG, under certain defined conditions, are effective in controlling and eliminating micrometastases in a syngeneic guinea pig tumor system, regardless of whether the animals are BCG immune or not. At the outset it should be stated that this experimental model has major limitations in that it is a transplantable tumor established for a short time in normal guinea pigs and in that the system out of necessity does not take into account such factors as individual variations between the biological behavior of tumors of other histological types and the limiting factors of the host. Thus, the model may be used to answer only specific questions fundamental to immunotherapy of micrometastasis.

Under natural conditions the development of metastasis is dependent upon an interplay between properties of the host and properties of the tumor cells. The process is highly selective and represents the end point of several destructive events from which few tumor cells survive. Only a few tumor cells within the primary neoplasm may actually invade blood vessels, and of those even fewer will survive in the circulation. Similarly, not all malignant cells that survive transport are successfully arrested, undergo extravasation, etc. Also, tumor cells, in principle, could be susceptible to host immune and nonimmune defense mechanisms that could destroy malignant cells during any of the steps described above (5, 6).

Metastasis was artificially induced in guinea pigs by i.v. injection of L10, and treatment was not started until adequate time had elapsed to ensure extravasation and localization of tumor cells into the parenchyma of visceral organs. No significant difference in the effectiveness of vaccines was found when the treatment was started 1 or 4 days after tumor cell transplantation. It has previously been demonstrated with i.v. injection of B16 melanoma in mice (4) that, between 1 and 4 hr after i.v. transplantation, there is a 50% reduction in the number of arrested tumor cells in the lung, and at 24 hr only 2% of the cells are retained in the lung as a stable metastatic population. Thus, the results of any treatment administered prior to 24 hr after transplantation are impossible to interpret since beneficial effects could be due to prevention of metastasis rather than to treatment. In this study the lack of difference between

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various effective vaccines when treatment was adminstered 1 or 4 days after transplantation suggests that a therapeutic effect was indeed achieved with these vaccinations.

It was clear from this study that, of the 2 basic BCG-tumor cell vaccines used, the preparation consisting of 10^6 viable BCG admixed with 10^7 tumor cells was more effective over a broad range of increasing initial tumor burdens than was that of 10^6 BCG admixed with 10^7 tumor cells. Although the latter was effective at initial tumor burdens at 10^4 L10, it was ineffective when the initial tumor cell inoculum was increased to 10^5 L10. Whether this 10:1 BCG:tumor cell ratio is critical or simply a function of total BCG cannot be determined from these studies. However, in a study of the effectiveness of BCG-tumor cell mixtures as vaccines against LSTRA murine leukemia (2), it was found that immunity was high (100%) if the BCG:LSTRA ratio was low (either 5×10^4:10^5 or 5×10^6:10^5) and that the proportion of immune mice was low (8%) if the BCG:LSTRA ratio was high (5×10^6:10^5).

Tumor cells that were frozen by an established procedure used for preservation of bone marrow in transplantation studies and assessed as an optimal procedure in several low-temperature biology studies (for review, see Ref. 24) were equally as effective in the vaccines as fresh tumor cells. This is contrary to the results of Bartlett *et al.* (1) who used glycerol as the freezing additive. Our cells were frozen in dimethyl sulfoxide and fetal calf serum. The striking difference, however, was the percentage of viability after freezing. Cell viability was approximately 90% after freeze-thawing. If for some reason viability fell below 80% during liquid nitrogen storage, the cells were discarded. In our opinion, the trypan blue exclusion test is a very conservative test of cell damage, and any trauma sufficient to render 20% of the cells sensitive to trypan blue may have severely damaged the remaining cells or altered their antigenicity. The viability of frozen cells in the experiments of Bartlett *et al.* ranged between 40 and 70% as determined by trypan blue exclusion. Thus, the difference in results with frozen L10 cells may be attributed to suboptimal *versus* optimal freezing conditions. Whether the 20,000-R X-irradiation dose of the tumor cells was an important aspect in the preparation of cells in vaccines is not known. However, studies are under way to test this point in this model since it is recognized that the use of 12,000 R is standard procedure for BCG-tumor cell vaccines in humans.

Of the 3 basic vaccination schedules tested, the 2 that were consistently effective for all tumor burdens were 10^6 BCG admixed with 10^7 L10 followed by 10^7 alone on the opposite side or 2 separate injections of 10^6 BCG admixed with 10^7 L10. The fact that BCG was not required in the second injection of the former schedule and the fact that multiple vaccinations did not improve therapy with respect to the latter schedule or with the less effective vaccine (10^6 BCG + 10^7 L10) suggest that the critical aspect of any of the vaccination schedules is the initial dose of BCG. Following the initial treatment with BCG, effective systemic tumor immunity can be achieved with i.d. injections of tumor cells alone at a different site.

A third vaccination schedule, which consisted of reintroduction of the tumor immunogen into the i.d. site previously injected with BCG + tumor cells, was effective at lower tumor burdens (10^4) but was less effective at initial tumor burdens of 10^5. This is in contrast to a similar schedule in which the second tumor cell vaccination was in a different i.d. site. The rationale for the second injection of tumor cells in the BCG-infected site was based on the study of Hawrylko (13), in which the dimensions of BCG-potentiated antitumor response against the murine mastocytoma P815 were investigated. One limitation that we found with this procedure was the difficulty in delivering the tumor cell inoculum in the previously infected dermal site. Early ulcerations of these injected dermal sites were limiting with respect to constant delivery of the tumor immunogen in the second injection.

In this study we have shown that visceral micrometastasis induced by i.v. injection of L10 can be cured by the systemic effect of a tumor cell-BCG vaccine. These results confirm our previous studies on the immunological susceptibility of i.v.-injected L10 cells (9, 10). We have now demonstrated that a nontumorigenic vaccine can affect immunotherapy. These results demonstrate that there is a critical dose for BCG in the initial vaccination but that BCG is not essential in the subsequent vaccination and that optimum therapy could be achieved with 2 vaccinations separated by a period of 6 days. Furthermore, the induced tumor immunity, which can cure the majority of guinea pigs with micrometastases is achieved by 2 vaccinations that require a total of 2×10^7 tumor cells (approximately 20 mg of tumor) administered over a period of 1 week. Also, the tumor cells, when frozen under established optimal conditions, maintain immunogenicity and can be used effectively in vaccines.

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Exhibit E

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..ABLISHMENT OF A TUMOR-SPECIFIC IMMUNOTHERAPY MODEL UTILIZING TNP-REACTIVE HELPER T CELL ACTIVITY AND ITS APPLICATION TO THE AUTOCHTHONOUS TUMOR SYSTEM[1]

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'reinduction of potent hapten-reactive helper T l activity and subsequent immunization with pten-coupled syngeneic tumor cells result in enced induction of tumor-specific immunity ough T-T cell collaboration between anti-hapten lper T cells and tumor-specific effector T cells. On basis of this augmenting mechanism, a tumor-ecific immunotherapy protocol was established which a growing tumor regresses by utilizing a tent trinitrophenyl (TNP)-helper T cell activity. H/He mice were allowed to generate the amplified ore potent) TNP-helper T cell activity by skin inting with trinitrochlorobenzene (TNCB) after treatment with cyclophosphamide. Five weeks er, the mice were inoculated intradermally with ng[illegible]c transplantable X5563 tumor cells. When s injected into X5563 tumor mass, an ap-ble number of growing tumors, in the only up of C3H/He mice in which the amplified TNP-lper T cell activity had been generated were ob-rved to regress (regressor mice). These regressor ce were shown to have acquired tumor-specific T ll-mediated immunity. Such immunity was more tent than that acquired in mice whose tumor was nply removed by surgical resection. These results licate that *in situ* TNP haptenation of the tumor lls in TNP-primed mice can induce the enchanced mor-specific immunity leading to the regression a growing tumor. Most importantly, the present dy further investigates the applicability of this P immunotherapy protocol to an autochthonous mor system. The results demonstrate that an ap-eciable percent of growing methylcholanthrene-duced autochthonous tumors regressed by the ove TNP immunotherapy protocol. Thus, the pres-t model provides an effective maneuver for tumor-ecific immunotherapy in syngeneic transplanta-as well as autochthonous tumor systems.

On the basis of the hypothesis of Mitchison (1) con-ming manipulations that might augment tumor-spe-ic immunity, numerous attempts to enchance the immunogenicity of tumor-associated transplantation antigens (TATA)[3] by coupling additional antigenic determinants on the tumor cell surface have been reported (2–6). Helper T cells can collaborate with effector T cell precursors, such as cytotoxic cell precursors, to enhance immune responses against various antigens including TATA (7). If additional determinants coupled onto the tumor cell act as helper determinants, it is therefore conceivable that preinduction of helper T cell activity to these additional determinants could induce much higher anti-TATA immune responses at the time of stimulation of tumor cells conjugated with the corresponding antigenic determinants.

We defined conditions under which enhanced immune resistance to tumors could be generated by preinducing trinitrophenyl (TNP) hapten-reactive T cells, and by subsequently immunizing with TNP-coupled syngeneic tumor cells (8, 9). This system is designed to induce the most efficient generation of tumor-specific effector T cell activity *in vivo* by virtue of the close linkage of hapten-reactive helper T cells and TATA-specific effector precursor T cells in the microenvironment at the time of stimulation with hapten-coupled tumor cells. Our previous results demonstrated that the generation of potent TNP-helper cell activity after elimination of suppressor cell activity was a prerequisite for amplified generation of *in vivo* protective immunity, and a T-T cell interaction mechanism between TNP-helper T cells and anti-TATA effector T cell precursors was thus suggested to be essential to such a phenomenon (10). These results prompted us to establish an immunotherapeutic protocol in tumor-bearing animals in which such potent TNP-helper T cells were used.

In the present study, when TNP was introduced into the tumor mass of tumor-bearing mice in which the amplified TNP-reactive helper T cell activity had been generated, *in situ* trinitrophenylation of tumor cells resulted in a high incidence of complete regression of growing tumors. We demonstrated that the tumor regression was accompanied by the concurrent generation of a potent tumor-specific T cell immunity, suggesting on the above T-T cell collaboration mechanism was functioning in this tumor immunotherapy protocol. More importantly, the present study also investigates whether such an immunotherapeutic potential realized in the TNP-helper

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[3] Abbreviations used in this paper: TATA, tumor-associated transplantation antigens; TNCB, trinitrochlorobenzene; MCA, 3-methylcholanthrene; Cy, cyclophosphamide; CTL, cytotoxic T lymphocyte; i.d., intradermal; DTH, delayed-type hypersensitivity.

TABLE I

Comparison of acquisition of tumor-specific immunity after TNP immunotherapy and surgical resection of tumor

Group	Mice	Incidence of Resistance against Tumor Challenge[a]
A	Normal	0/10
B	After regression of tumor by TNP immunotherapy[b]	11/12
C	After surgical resection of tumor[c]	2/10

[a] Mice were challenged i.d. with 10^6 viable X5563 tumor cells, and incidence of resistance was determined 3 weeks after the tumor challenge.

[b] C3H/He mice whose X5563 tumor regressed in the TNP immunotherapy model as shown in Fig. 1 (group E) were used 3 wk after initial tumor implantation.

[c] C3H/He mice were inoculated i.d. with 10^6 X5563 tumor cells and growing tumors were surgically resected 7 days later. Mice were used 2 wk after tumor removal.

growth of metastasized tumor cells. Thus, the difference in incidence of anti-X5563 immunity between two groups above indicates more potent anti-X5563 immune resistance was retained in mice whose tumors regressed by virtue of the TNP immunotherapy.

The development of stronger anti-X5563 immune resistance in regressor mice was also confirmed by comparing the tumor-neutralizing activity of spleen cells from these mice to that of mice whose tumors were surgically resected. Winn assay performed with these two groups of spleen cells at a lower spleen to tumor cell ratio [illegible] that appreciably stronger tumor-neutralizing activity was generated in the regressor mice by TNP immunotherapy than in the mice that had tumors resected surgically (Table II).

Additional experiments were performed to test the nature and specificity of the effector mechanism acquired by X5563 tumor regressor mice. Winn assays with the use of spleen cells from the regressor mice also demonstrated that these spleen cells resulted in complete neutralization of X5563 tumor cells when admixed, but failed to exhibit 1) tumor neutralization against X5563 tumor after the treatment of the spleen cells with anti-Thy-1.2 plus C (Table III), and 2) tumor neutralization against another syngeneic tumor MH134 hepatoma (Table IV). These results indicate the T cell nature and specificity of anti-X5563 immunity acquired by the regressor mice in the TNP immunotherapy model.

Application of the TNP immunotherapeutic protocol to an autochthonous tumor system. In the process of application of the present tumor-specific immunotherapy model to a chemical carcinogen-induced autochthonous tumor system, we extended this TNP immunotherapy model to another transplantable, chemical carcinogen-induced tumor (MCH-1-A1) system in which the tumor was recently induced in C3H/He mice by MCA and has been maintained in our laboratory (less than 10 passages *in vivo*). A similar protocol to that performed in the X5563 tumor system was used and the results are illustrated in Figure 2. In this experiment, TNCB injection into the MCH-1-A1 tumor mass from Cy→TNCB-painted mice led to a high incidence of tumor regression, in contrast to the lack of tumor regression when *in situ* TNP modification was performed in mice not primed to TNP. Thus, this TNP immunotherapy system is also applicable to another recently established transplantable, chemical carcinogen-induced fibrosarcoma tumor system.

The successful regression of growing tumors in an MCA-induced transplantable tumor system by using the TNP immunotherapy regimen encouraged us to test the applicability of this immunotherapy protocol to an MCA-induced autochthonous tumor system. The primary tumor was induced in 500 female C3H/HeN mice at 8 wk of age by injecting 0.5 mg MCA in 0.1 ml olive oil subcutaneously. Four weeks after the MCA inoculation, one half of the group of mice received the combined treatment of Cy injection and TNCB painting, which was capable of inducing the amplified TNP-reactive helper T cell activity, and the remainder were untreated. The mice began to develop a primary, subcutaneous tumor about 8 wk after the MCA treatment. At 9 wk after the MCA injection, 20 to 30% of mice in both TNP-helper-positive and -negative groups bore a tumor in the range of 6 to 9 mm in diameter. Histological examination of 10 autochthonous tumors randomly selected (five mice in each group) revealed that all were fibrosarcoma. Mice that did not receive tumor excision were collected and each group was randomly divided into two groups depending on whether mice were treated with the intratumoral injection of 0.15 ml of 1% TNCB. Therefore, the experiment consisted of four groups: group A: MCA injection only; group B: MCA injection→intratumoral TNCB injection; group C: MCA injection→the combined treatment of Cy plus TNCB painting; and group D: MCA injection→the above combined treatment for priming of potent TNP-helper T cells→intratumoral TNCB injection. The tumor growth of four groups of animals is shown in Figure 3. Most tumors in three groups of mice (groups A, B and C), except for only one animal in group B, continued to grow until the animal died, although the growth rate exhibited varied patterns. Importantly, however, an appreciable number (11 of 25)

TABLE II

Comparison of tumor-neutralizing activity between spleen cells from mice after tumor regression after TNP immunotherapy and from mice after surgical resection of tumor

Spleen Cells from Mice	Spleen:Tumor Cell Ratio	Tumor Growth[a] (mm diam)		
		Day 7	Day 10	Day 12
[illegible]tion of tumor by TNP immunotherapy[b]	100:1	5.8 ± 1.3	9.6 ± 1.0	13.5 ± 0.3
[illegible]al resection of tumor[c]	100:1	<3.0	<3.0	<3.0
	100:1	<3.0	<3.0	<3.0
After regression of tumor by TNP immunotherapy	10:1	4.8 ± 0.9	9.3 ± 0.9	12.5 ± 1.0
After surgical resection of tumor	10:1	<3.0	<3.0	5.0 ± 1.5
	10:1	<3.0	7.5 ± 0.5	10.3 ± 1.2

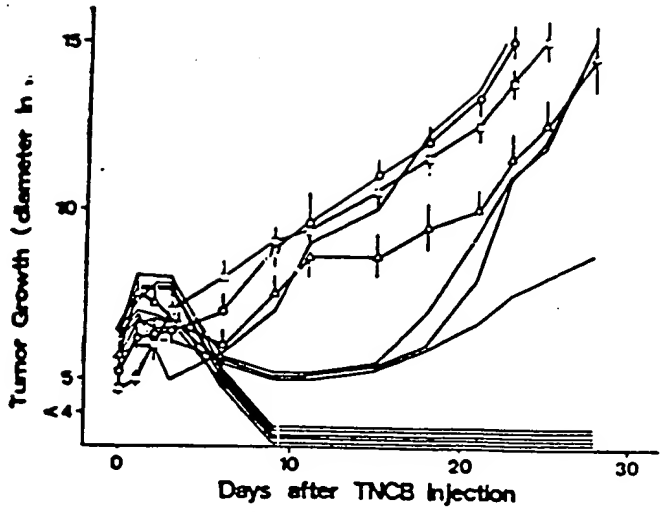

Figure 2. Induction of tumor regression in MCH-1-A1 tumor-bearing mice by using the TNP immunotherapy regimen. C3H/He mice received the combined treatment of Cy injection and TNCB painting. Five weeks after TNCB painting, mice were inoculated i.d. with 10^6 viable MCH-1-A1 tumor cells. The *in situ* TNP haptenation identical to that in Fig. 1 was performed 7 days after tumor cell inoculation. Tumor growth was individually scored and expressed by tumor diameter (——). Tumor growth in control groups was expressed by mean diameter ± SE of seven mice per group. (○——○), (△——△), and (□——□) indicate tumor cell inoculation only, tumor cell inoculation→*in situ* TNP haptenation, and the above combined treatment for TNP priming→tumor cell inoculation, respectively.

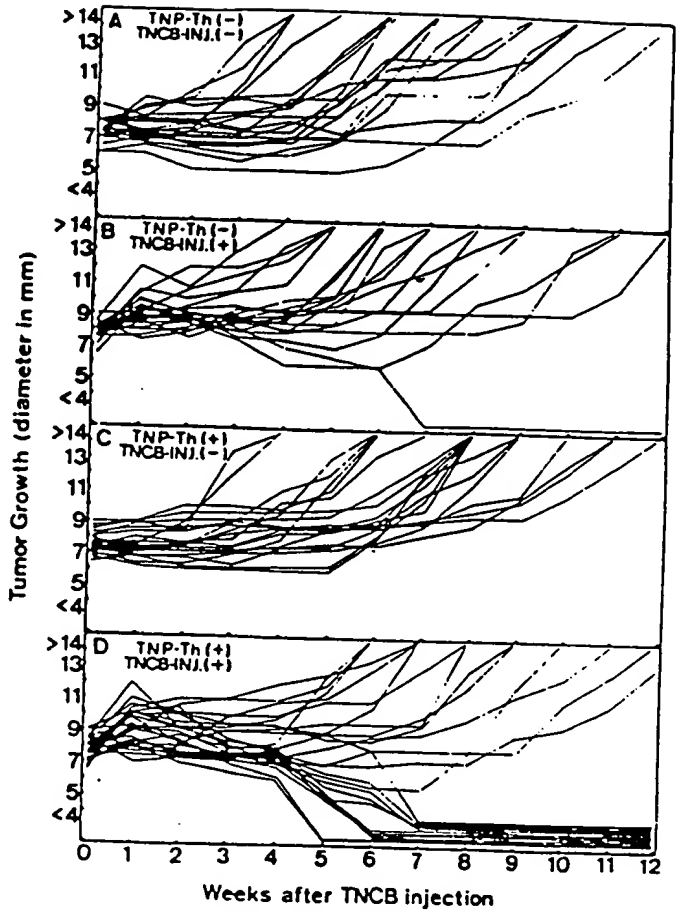

Figure 3. Regression of growing autochthonous tumors by the TNP immunotherapy regimen. C3H/He mice were inoculated subcutaneously with 0.5 mg MCA. Four weeks later, mice received the combined treatment of Cy injection and TNCB painting (groups C and D). Nine weeks after MCA injection, 0.15 ml TNCB in olive oil was administered into subcutaneously growing autochthonous tumors of groups B and D. Group A was MCA inoculation only. Tumor growth was individually scored and expressed by tumor diameter. Tumors in all groups that reached a 14-mm diameter continued to grow for as many as 8 wk ultimately killing the animal. For limitation of the scale, such stage of growth was omitted.

TABLE V

Summary of incidence of tumor regression and mean survival time[a]

Group	Treatment		Incidence of Tumor Regression	Mean Survival Time (weeks ± SE)	No. Dead Mice
	TNP-Th induction	TNCB injection			
A	−	−	0/20	13.00 ± 0.75	20
B	−	+	1/20	11.32 ± 0.55	19
C	+	−	0/20	13.60 ± 0.58	20
D	+	+	11/25	12.90 ± 0.68	14

[a] Determined 20 wk after injection of TNCB into autochthonous tumor and expressed by mean survival time of dead mice at this stage.

T cell immunity was more potent in the tumor-regressed than in mice whose tumor was surgically resected.

ould also be noted that X5563 tumor-specific im-unity, which had been acquired in tumor-regressed mice, was mediated by anti-X5563-TATA-specific Lyt-1$^+$2$^-$, but not by Lyt-1$^-$2$^+$ T cells, indicating that the tumor-specific Lyt-1$^+$2$^-$ T cell population whose generation was augmented through collaboration with TNP-specific helpers primarily exhibited a protective effect (T. Yoshioka, H. Fujiwara, and T. Hamaoka, manuscript in preparation). Because these Lyt-1$^+$2$^-$ T cells exhibited no cytotoxic effect on X5563 tumor cells in a 4-hr ^{51}Cr-release assay, further studies are in progress concerning the mechanisms of anti-tumor-specific Lyt-1$^+$ T cell function in eradicating tumor cells *in vivo*.

The most interesting and important finding in the present study [which has not been reported in other tumor-specific immunotherapy experiments] is the demonstration of the applicability of this TNP immunotherapy protocol to an autochthonous tumor system. This finding is worthy of discussion from two perspectives. First, the evidence that TNP immunotherapeutic potential allows the induction of tumor regression to an appreciable proportion in autochthonous as well as transplantable tumor systems clearly emphasizes the validity of this TNP immunotherapy model on the basis of the T-T cell interaction mechanism. This could also provide a theoretical basis for Klein's clinical approaches in which skin malignancies were treated by haptenic reagents (16). Although further investigation is required to explore a chemical

t suitable for the *in situ* modification of human ...rs, the present system may provide a prototye of the immunotherapy of some types of clinical tumors such as skin cancers.

Second, it remains to be proven why 14 of 25 of the autoch... been assumed that most of tumors bear TATA (17, 18), the qualitative diversity and quantitative heterogeneity in the expression of each putative TATA on an autochthonous tumor cell has not been well determined. Further experiments are therefore required to determine whether the tumor-specific immunity is in fact acquired in mice whose autochthonous tumor has regressed and how putative TATA in each individual autochthonous tumor qualitatively varies, and to investigate the relationship between the immunogenicity of the autochthonous tumor and the prognosis of the tumor-specific immunotherapy. Such approaches are in progress by challenging the autochthonous tumor cells obtained by excisional biopsy

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COMMUNICATION

The Induction of Cytolytic T Lymphocytes with Syngeneic Trinitrophenyl-Coupled Membranes[1]

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Recently we have demonstrated the induction of allogeneic murine cytolytic T lymphocytes (CTL)[3] using purified plasma membranes rather than intact cells as stimulatory agents (1). In this report we extend the use of such subcellular preparations to study the requirements for hapten-specific syngeneic CTL induction. Membranes prepared from trinitrophenyl (TNP) coupled syngeneic tumor cells retain the ability to stimulate both a primary and secondary CTL response. The CTL that are generated are restricted in their lysis to target cells bearing the same H-2 antigens as those present on the TNP-coupled stimulating membranes.

MATERIALS AND METHODS

All materials and methods used in the *in vitro* induction and assay of TNP specific CTL are as previously described (2). Briefly, 7×10^6 spleen cells from nonimmune or immune mice were co-cultured with x-irradiated, TNP-coupled spleen cells or TNP-coupled membranes. After 5 days of culture cells were harvested and cytolytic activity was assessed in a 4-hr assay against 10^4 ^{51}Cr-labeled TNP-coupled tumor targets or LPS blast cell targets. Immune spleen cells were obtained by priming mice subcutaneously with 2×10^7 TNP-coupled autologous spleen cells 2 weeks before *in vitro* culture. Membranes used in stimulation of CTL were prepared from TNP coupled DBA/2 mastocytoma P815 (H-2^d) tumor cells or from TNP-coupled C57BL/6 (B6) leukemia EL-4 (H-2^b) tumor cells. Purified plasma membranes were used for CTL induction in the experiment described in Table I. The results presented in Tables II and III were obtained by using partially purified plasma membranes referred to as "high speed pellet" in Reference 1. Spontaneous ^{51}Cr release ranged from 30 to 39% for LPS-induced blast cell targets and from 11 to 19% for tumor cell targets.

RESULTS

Plasma membranes prepared from TNP-modified tumor cells were tested for their ability to stimulate both primary and secondary CTL responses by using H-2 syngeneic responder cells. As demonstrated in Tables I and II, such membranes were active in the stimulation of primary and secondary hapten specific CTL. CTL generated by coupled membranes are similar in specificity to those generated by coupled cells in that they preferentially lyse syngeneic target cells (2–4). These membranes stimulated variable amounts of cross-reactive lysis on TNP coupled allogeneic target cells. B6 spleen cells stimulated with TNP-EL-4 membranes did not lyse targets that did not bear TNP. Lytic activity was not induced when B6 spleen cells were co-cultured with uncoupled EL-4 membranes.

It has been recently demonstrated (5, 6) that cells incubated with TNP-coupled proteins are capable of stimulating a hapten-specific cytolytic response that is restricted to target cells that are H-2 identical with the responder cell population. Therefore, it was important to determine if stimulation by the membranes was dependent on the H-2 antigen present on the membrane, or whether the membrane proteins were simply contributing the hapten that was then recognized in conjunction with the H-2 antigens of the responder cell population. B6D2F$_1$ (H-$2^{b/d}$) immune spleen cells were stimulated with either TNP-EL-4 membranes or TNP-P815 membranes and the specificity of the resultant CTL was studied. It would be expected that if the H-2 present on the membranes did not influence the specificity of the CTL, then in either case the CTL would lyse both B6-TNP (H-2^b) and B10.D2-TNP (H-2^d) targets to a similar extent. As is shown in Table III the CTL preferentially lyse target cells that bear the same H-2 antigens as the TNP-membranes used in CTL stimulation. Similar specificity was obtained with CTL resulting from stimulation of nonimmune B6D2F$_1$ spleen cells (Table II). These results indicate that both the TNP and the H-2 antigens present on the membranes determine the specificity of the CTL population.

DISCUSSION

The results described above extend the use of subcellular material to the study of CTL recognition in a chemically modified syngeneic system. The results demonstrate the capacity of membranes prepared from TNP-modified tumor cells to induce primary and secondary CTL having the same specificity as CTL that are induced by TNP-coupled cells. The ability to stimulate B6D2F$_1$ CTL that are restricted in their recognition to the H-2 antigens present on the stimulating membrane

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[3] Abbreviations used in this paper: CTL, cytolytic T lymphocytes; H-2, histocompatibility complex-2; B6D2F$_1$, (C57BL/6 × DBA/2)F$_1$; B6, C57BL/6.

TABLE I

Specificity of secondary (BALB/c × DBA/2)F₁ (H-2ᵈ) CTL stimulated by TNP-coupled membranes

Stimulator[a]	% Specific ^{51}Cr Release			
	P815-TNP (H-2ᵈ)		EL-4-TNP (H-2ᵇ)	
	25/1[b]	12.5/1	25/1	12.5/1
Experiment 1				
—	19	9		
12-μg membranes	32	19		
24-μg membranes	44	24		
72-μg membranes	59	38		
Experiment 2				
—	31	19	19	12
BALB/c-TNP cells	83	61	40	14
3-μg membranes	31	16	13	8
10-μg membranes	41	19	17	11
30-μg membranes	63	40	22	14

[a] Membranes used for this experiment were purified plasma membranes obtained from TNP-coupled P815 tumor cells (1).

[b] Effector to target ratio.

TABLE II

Induction of primary (C57BL/6 × DBA/2)F₁ (H-2ᵇ/ᵈ) CTL by TNP-coupled membranes

Stimulator[a]	% Specific ^{51}Cr Release[b]	
	B6-TNP (H-2ᵇ)	B10.D2-TNP (H-2ᵈ)
—	7	7
B6-TNP cells	64	34
24-μg membranes	28	12
75-μg membranes	33	6
150-μg membranes	38	6

[a] Membranes used in this experiment were partially purified from TNP-coupled EL-4 tumor cells (H-2ᵇ).

[b] Effector to target ratio is 50:1. Target cells were LPS-stimulated blast cells.

TABLE III

Specificity of TNP-membrane-induced CTL

Responder	Stimulator	% Specific ^{51}Cr Release Targets[a]	
		B6-TNP (H-2ᵇ)	B10.D2-TNP (H-2ᵈ)
Primed B6D2F₁ (H-2ᵇ/ᵈ)	—	6	9
	75 μg EL-4-TNP membranes	29	10
	84 μg P815-TNP membranes	16	32

[a] Effector to target ratio was 50:1. Targets were LPS-stimulated blast cells.

preparations indicates that induction results from recognition of both the H-2 and the hapten on the membranes and not from haptenated protein(s) from the membranes that associate with the responder cells. In this regard, TNP membranes are similar in their inductive capacity to TNP cells. It has been previously reported that H-2 antigens need not be directly haptenated in order to obtain a CTL response. Recent experiments that have utilized TNP-coupled serum proteins to stimulate TNP-specific CTL have argued against the contention by Forman *et al.* (7) that only TNP present on H-2 is antigenically active. Although the experiments described above do not address the question of a requirement for direct haptenization of H-2 to stimulate, it is clear that TNP-membranes are antigenically similar to TNP-cells rather than TNP-proteins.

It is also of interest to consider these results as they address the mechanism of CTL stimulation by subcellular material. The possibility exists that stimulation of CTL by subcellular preparations occurs via presentation of antigen by intact cells present in the cultures (e.g., macrophages) rather than by direct interaction between the membrane vesicle and pre-CTL. It is clear that if indeed material must be presented by viable cells to be antigenic, these cells do not determine the specificity of the resulting CTL.

Ozata and Henney (6) have reported that membranes from TNP-coupled spleen cells failed to induce a secondary syngeneic CTL response whereas the results shown in Tables I and II of this report clearly show that membranes from TNP-coupled tumor cells can induce a specific secondary response. This discrepancy might be accounted for by the difference in cell type used as a membrane source.

The ability to stimulate CTL with TNP-modified membranes opens the possibility that we will be able to isolate, in a soluble form, TNP-modified membrane proteins that retain biologic activity (i.e., the ability to induce CTL) (8). One could then determine whether an effective immunogen is created by TNP-modified non-MHC proteins that interact with H-2, or whether direct chemical modification of H-2 antigens creates the immunogen, or whether both possibilities exist.

SUMMARY

Evidence is presented that trinitrophenyl-coupled tumor membranes are able to induce cytolytic T lymphocytes (CTL) when co-cultured with syngeneic spleen cells. These haptenated membranes stimulate spleen cells from naive and immune mice. The specificity of these CTL is determined by the H-2 antigens of the membranes used for stimulation.

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